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Abiotic Stress Responses in Photosynthetic Organisms

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ABIOTIC STRESS RESPONSES IN PHOTOSYNTHETIC ORGANISMS

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ABIOTIC STRESS RESPONSES IN PHOTOSYNTHETIC ORGANISMS

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Cellular and molecular aspects of abiotic stress responses in *Arabidopsis thaliana* subjected to cold, drought, and high salinity and in two photosynthetic green alga, *Chlamydomonas reinhardtii* and *Coccomyxa* sp. C-169, subjected to nitrogen deprivation were investigated. Cold, drought, and high salinity can negatively affect plant growth and crop production. The first research aimed at determining the physiological functions of the stress-responsive *Arabidopsis thaliana RD29A* and *RD29B* genes. Cold, drought, and salt induced both genes; the promoter of *RD29A*was found to be more responsive to drought and cold stresses, whereas the promoter of *RD29B* was highly responsive to salt stress. Therefore, *RD29A* and *RD29B* gene sequences have the potential to confer abiotic stress resistance in crop species grown in arid and semi-arid regions. RD29A and RD29B proteins were also found to unlikely serve directly as protective molecules. The second study aimed at investigating the impacts of nitrogen deprivation in *Chlamydomonas reinhardtii* and *Coccomyxa* sp. C-169; results showed that these microalgae altered their lipid metabolism by synthesizing and accumulating the neutral lipid triacylglycerol (TAG). Since microalgae have emerged as suitable feedstocks for renewable biofuel production, the purpose of this analysis was to understand the genetic and biochemical mechanisms associated with the induction of TAG synthesis in *Chlamydomonas* and *Coccomyxa* subjected to nitrogen deprivation under photoautotrophic conditions. In

addition to TAG accumulation, nitrogen depletion triggered an early synthesis of starch and up-regulation of several genes in *Chlamydomonas*, including some diacylglycerol:acyl-CoA acyltransferases, catalyzing the acylation of diacylglycerol to TAG. Protein degradation in nitrogen-deprived cells might provide carbon skeletons for TAG biosynthesis. In a related study, the effects of the autophagy-inducer rapamycin and the autophagy-inhibitor 3-methyladenine (3-MA) on the accumulation of TAG in *Chlamydomonas* cells subjected to nitrogen deprivation were investigated. 3-MA induced TAG accumulation in cells growing in both nitrogen-deprived and control media. The increase in TAG content in cells subjected to nitrogen deprivation might not be a direct response to an autophagic activity induced by nutrient depletion.

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TO GOD BE THE GLORY!

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CHAPTER 1 - INTRODUCTION

Adaptation to unavoidable environmental stress is crucial for the survival of all living organisms. Under extreme abiotic stress conditions, organic molecules such as lipids, proteins, and nucleic acids are prone to damage and/or degradation (Miranda, 2011). The cellular and molecular basis of microbial and plant resistance to environmental stress have been studied intensively (Thomashow, 1999; Hasegawa et al., 2000; Xiong et al., 2002, Msanne et al., 2011a) and regulation of gene expression during abiotic stresses like cold, drought, high salinity, and lack of nutrients has been examined in several microorganisms (Yamamori and Yura, 1982; Neidhardt et al., 1983; Borbely et al., 1985; Berg et al., 1987; Msanne et al., 2011b), plants (Schlessinger et al., 1982; Ericson and Alfinito, 1984; Kimpel and Key, 1985; Ramagopal, 1987; Msanne et al., 2011a), and animals (Schlessinger et al., 1982; Apte and Bhagwat, 1989). Understanding the mechanisms by which microorganisms and plants perceive environmental signals and transmit the signals to cellular machinery to activate adaptive responses is of fundamental importance to biology (Xiong et al., 2002).

Cold, drought, and high salinity are common stress conditions that adversely affect plant growth and crop production (Xiong et al., 2002). Under these conditions, stress response mechanisms are activated, either to prevent the source of damage or to promote the rapid turnover of damaged molecules (Helder, 2011). Knowledge about stress signal transduction is vital to improve stress tolerance in crops (Xiong et al., 2002). Cold, drought, and high salinity are complex stimuli and it is unlikely that there is only one sensor that perceives the stress condition and controls all subsequent signaling (Xiong et al., 2002). Histidine kinases are involved in the transduction of environmental

signals in prokaryotes, protozoa, fungi, as well as in plants (Marin et al., 2003). These kinases can act as sensory molecules via a sensory domain, and a His residue is phosphorylated in response to changes in environmental conditions. The phosphoryl group is then transferred to a response regulator, a transcription factor, and the resultant activation of the response regulator controls the expression of a large number of downstream genes (Koretke et al., 2000).

Salinity is one of the most important abiotic factors limiting productivity in many arid and semi-arid environments around the globe (Msanne et al., 2011a). Although the molecular basis of the mechanisms involved in salt tolerance in many organisms is not fully understood, some concepts have emerged in recent years (Apte and Bhagwat, 1989). Plants have evolved complex mechanisms that contribute to the adaptation to osmotic and ionic stress caused by high salinity (Meloni et al., 2004). These mechanisms include (i) curtailment of Na^+ influx and prevention of intracellular Na^+ accumulation resulting in greater salt tolerance (Apte and Thomas, 1986; Apte et al., 1987; Apte and Bhagwat, 1989); (ii) osmotic adjustment that is usually accomplished by uptake of inorganic ions, such as K^+ (Miller et al., 1976; Thomas and Apte, 1984; Reed and Stewart, 1985; Apte and Bhagwat, 1989; Meloni et al., 2004), as well as the accumulation of compatible solutes (osmoprotectants), such as glucopyranosylglycerol (Borowitzka et al., 1980), sucrose (Blumwald et al., 1983), trehalose, or glycine betaine (Reed et al., 1984). Inorganic ions are sequestered in the vacuoles (Binzel et al., 1988), while organic solutes are compartmentalized in the cytoplasm to balance the low osmotic potential in the vacuole (Rontein et al., 2002) and (iii) metabolic adjustments to tune the cellular activities to function at higher internal osmoticum (Blumwald and Tel-Or, 1983; Thomas

and Apte, 1984.). Osmoprotectants are small, nontoxic, electrically neutral molecules that stabilize proteins and membranes against the denaturating effect of high concentrations of salts and other harmful solutes (Munns, 2002; Meloni et al., 2004). Glycinebetaine acts as a compatible solute by stabilizing the quaternary structures of proteins, cell membranes, and the oxygen-evolving complex of PSII (Papageorgiou and Murata, 1995). The expression of genes that are involved in the biosynthesis of compatible solutes is enhanced under salt stress (Marin et al., 2006), and concentrations of accumulated compatible solutes are correlated with the extent of tolerance to salt stress (Zhu, 2002; Allakhverdiev and Murata, 2008).

Microorganisms like cyanobacteria have also evolved a variety of protective mechanisms against unfavorable salt-stress conditions that emphasize on the role of Na^+/H^+ antiporters (Allakhverdiev et al., 1999; Inaba et al., 2001), water and ionic channels (Allakhverdiev et al., 2000a; b), the synthesis of compatible solutes (Hagemann and Erdmann, 1997; Hayashi and Murata, 1998; Chen and Murata, 2002) and of salt stress-induced proteins (Bhagwat and Apte 1989; Hagemann et al., 1990; 1991; Allakhverdiev and Murata; 2008).

In order to cope with the unfavorable conditions such as lack of bioavailable nitrogen, certain algal species have developed a number of adaptations, including alteration of lipid metabolism and synthesis of nonmembranous lipids such as triacylglycerols (TAG) (Thompson, 1996; Cohen, 1999; Bigogno et al., 2002; Solovchenko et al., 2008) and carotenoids (Demmig-Adams et al., 1996; Rabbani et al., 1998; Ladygin, 2000). These mechanisms are described in chlorophytes such as *Chlamydomonas reinhardtii* (Msanne et al., 2011b), *Dunaliella salina* (Mendoza et al.,

1999), *Dunaliella bardawil* (Rabbani et al., 1998), *Haematococcus pluvialis* (Zhekisheva et al., 2002; Wang et al., 2003), and *Parietochloris incise* (Khozin-Goldberg et al., 2005; Merzlyak et al., 2007). Although nitrogen deficiency appears to inhibit the cell cycle and the production of almost all cellular components, the rate of lipid synthesis remains higher, which leads to the accumulation of neutral lipids in starved cells (Sheehan et al., 1998; Rosenberg et al., 2008). The TAG accumulated is often deposited in cytoplasmic lipid globules referred to as oil bodies (OB), which vastly increase in size and number under mineral nutrition deficiency (Bigogno et al., 2002), high salinity (Siaut et al., 2011), and high irradiances (Merzlyak et al., 2007). Lipids, particularly TAG, are thought to be a depot for the excessive photosynthates, which could not be utilized under unfavorable conditions (Bigogno et al., 2002). Nitrogen deprivation also promotes the accumulation of the antioxidant pigment astaxanthin in the green alga *Haematococcus pluvialis* (Boussiba, 2000). These adaptive responses help to ensure the cells' survival during times of stress; while lipids serve as energy stores, astaxanthin seems to play a role in the photoprotective functions against reactive oxygen species inevitably formed in the course of photosynthesis (Asada, 1994; Niyogi, 1999), quenching of chlorophyll (Chl) excited states (Demmig-Adams et al., 1996; Ladygin, 2000; Bukhov, 2001), and dissipation of the excess of absorbed light energy into heat (Lichtenthaler, 1987; Demmig-Adams et al., 1996; Havaux and Niyogi, 1999; Ladygin, 2000).

The overall objective of this dissertation is to analyze the effects of environmental stresses; i.e. cold, drought, high salinity, and lack of nitrogen on the following photoautotrophic organisms: *Arabidopsis thaliana*, *Chlamydomonas reinhardtii* and *Coccomyxa* sp. C-169. I focused my investigations on the physiological characterization

of two well-known *Arabidopsis thaliana* stress-responsive genes (*RD29A* and *RD29B*) under cold, drought, and high salinity conditions in a way that will allow us to determine the utility of these gene sequences for genetically engineering crops with improved field performance (Chapter 2); I also analyzed the genetic and biochemical mechanisms associated with the induction of triacylglycerol synthesis in *Chlamydomonas reinhardtii* and *Coccomyxa* sp. C-169, under nitrogen deprivation, when grown in liquid media (Chapter 3); and determine the effects of autophagy-inducing and autophagy-inhibiting compounds on the accumulation of triacylglycerol in the green algae *Chlamydomonas reinhardtii* (Chapter 4).

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CHAPTER 2 - CHARACTERIZATION OF ABIOTIC STRESS-RESPONSIVE *Arabidopsis thaliana RD29A* and *RD29B* GENES AND EVALUATION OF TRANSGENES

Abstract

Abiotic stresses have adverse effects on plant growth and productivity. The homologous *RD29A* and *RD29B* genes are exquisitely sensitive to various abiotic stressors. Therefore *RD29A* and *RD29B* gene sequences have potential to confer abiotic stress resistance in crop species grown in arid and semi-arid regions. To our knowledge, no information on the physiological roles of the proteins encoded by *RD29A* and *RD29B* are available in the literature. To understand how these proteins function, we used reverse genetic approaches, including identifying *rd29a* and *rd29b* T-DNA knockout mutants, and examining the effects of complementing transgenes with the genes under control of their native promoters and chimeric genes with the native promoters swapped. Four binary vectors with the *RD29A* and *RD29B* promoters upstream of the cognate *RD29A* and *RD29B* cDNAs and as chimeric genes with noncognate promoters were used to transform *rd29a* and *rd29b* plants. Cold, drought, and salt induced both genes; the promoter of *RD29A* was found to be more responsive to drought and cold stresses, whereas the promoter of *RD29B* was highly responsive to salt stress. Morphological and physiological responses of *rd29a* and *rd29b* plants to salt stress were further investigated. Root growth, and photosynthetic properties declined significantly, while solute concentration ($\Psi\pi$), water use efficiency (WUE) and $\delta^{13}C$ ratio increased under salt stress. Unexpectedly, the *rd29a* and *rd29b* knockout mutant lines maintained greater root

growth, photosynthesis, and WUE under salt stress relative to control. We conclude that the RD29A and RD29B proteins are unlikely to serve directly as protective molecules.

Keywords: Abiotic stress, *RD29*, T-DNA knockouts, gas exchange, osmotic potential, carbon isotope ratio.

Introduction

Plant growth and productivity are greatly affected by abiotic stresses, such as cold, drought, and salinity (Kasuga et al., 1999; Sakuma et al., 2006). Plants respond to these stresses through various biochemical and physiological processes, including decreased stomatal conductance, carbon fixation and efficiency of light harvesting mechanism, repression of cell growth, alteration in enzymes activity, and increased respiration and accumulation of osmolytes and proteins involved in stress tolerance (Yamaguchi-Shinozaki and Shinozaki, 1993; Shinozaki et al., 2003; Sakuma et al., 2006; Shinozaki and Yamaguchi-Shinozaki, 2007). At the transcriptional level, two major groups of genes have been identified to respond to cold, drought, salt stress, or a combination thereof (Thomashow, 1999; Bray et al., 2000; Shinozaki et al., 2003). The first group of gene products directly protects against stresses through synthesis of proteins that function by protecting cells from dehydration. This group includes enzymes involved in the synthesis of various osmoprotectants like the late embryogenesis abundant (LEA) proteins, osmotin, chaperones, sugar and proline transporters, detoxification enzymes, and various proteases (Shinozaki and Yamaguchi-Shinozaki, 2007). The second group regulates gene expression and signal transduction pathways

(Kaur and Gupta, 2005), and includes transcription factors (TFs), protein kinases like mitogen-activated protein (MAP) kinases, calcium-dependent protein kinases (CDPKs), receptor-like kinases, and histidine kinases (Xiong et al., 2002; Zhu, 2002; Shinozaki et al., 2003). Transcription factors such as the Abscisic Acid (ABA)-Responsive Element Binding Protein (AREB) and the Dehydration-Responsive Element-Binding Protein (DREB) play an important role in plant abiotic stress responses; they are involved in signal transduction by binding to the Abscisic Acid-Responsive Element (ABRE; PyACGTGGC) motif, a major *cis*-acting element found in the promoter region of ABA-inducible genes (Fujita et al., 2005), and the Dehydration Responsive Element (DRE/CRT; A/GCCGAC), respectively (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger et al., 1997; Gilmour et al., 1998; Liu et al., 1998; Shinwari et al., 1998; Sakuma et al., 2002; Dubouzet et al., 2003; Shinozaki et al., 2003; Cook et al., 2004; Gilmour et al., 2004; Novillo et al., 2004; Shinozaki et al., 2007; Wang et al., 2008).

Many stress-inducible genes contain both ABRE and DRE/CRT motifs in their promoters; these *cis*-acting elements function in ABA-dependent and ABA-independent gene expression in response to abiotic stress, respectively (Thomashow, 1999; Bray et al., 2000; Xiong et al., 2002; Shinozaki et al., 2003). Yamaguchi-Shinozaki et al., (1992) cloned and characterized nine independent cDNAs (named *RD*) whose corresponding genes are *R*esponsive to *D*esiccation stress in *Arabidopsis thaliana*. One of the *RD* clones, *RD29*, was reported to be quickly and strongly induced by drought and salt stresses. Genomic DNA southern blot analysis indicated that there are two closely related genes, *RD29A* and *RD29B,* that couldn't be distinguished by RNA blot analysis. These

genes are tandemly located in an 8 kb region of the *Arabidopsis* genome (Yamaguchi-Shinozaki and Shinozaki, 1993). The promoter region of *RD29A* with several DREs and one ABRE is mainly induced through the ABA-independent pathway, while *RD29B* promoter region carrying several ABRE sequences is controlled mainly by ABA, according to analyses in the ABA-deficient and -insensitive mutants *aba1* and *abi1*, respectively (Koornneef et al., 1984; Koornneef et al., 1992; Yamaguchi-Shinozaki and Shinozaki, 1994; Fujita et al., 2005). Both *RD29A* and *RD29B* genes encode closely related hydrophilic proteins of unknown function, appear to be limited to the *Brassicaceae* family and were named for responsive to desiccation, but have subsequently been shown to be differentially responsive to cold, drought, and salt stresses resulting in alternative designations such as *COR* (Cold-regulated) and *LTI* (Low temperature induced). *RD29A* and *RD29B* appear to be tandem gene duplications and encode very similar proteins (Yamaguchi-Shinozaki and Shinozaki, 1993).

This paper characterizes the abiotic stress-responsive *Arabidopsis thaliana RD29A* and *RD29B* genes. No close homologs are identified in relevant crop species, such as maize, rice, sorghum, and soybean. Efforts to understand the functions of these genes have focused on the responsiveness of the promoters to abiotic stresses, and to our knowledge there is no information available in the literature or reported on the physiological roles of the encoded proteins. The main objectives of this research are therefore: To characterize *rd29a* and *rd29b* knockout mutants and transgenics to determine physiological functions under stress, and to assess *RD29A* and *RD29B* promoters, cDNAs, and proteins to determine the utility in engineering abiotic stress resistance and improving crop productivity.

We focused the physiological and morphological study on salt stress because, firstly salt greatly induced the expression of *RD29A* and *RD29B* genes, and secondly salt is one of the most important abiotic factors limiting productivity in many arid and semi-arid environments around the globe. Furthermore, the problem of salinity is expected to intensify with the increase in drought frequency under projected climate change scenarios in many irrigated agricultural areas. Many of these areas are already experiencing salinity problems in soil and ground water (Lambers, 2003; Koyro, 2006). The approach of developing salt tolerant crops through genetic engineering has gained significant attention in the last decades (Koyro, 2006). Salt stress can be detrimental to plants. As plants exposed to salinity stress suffer osmotic stress injuries due to decreased soil water potential, ion toxicities resulting from accumulation of Na^+, Cl^- or SO_4^{-2} in the cells, and ionic imbalance due to competition of Na^+ with Ca^{2+} which results in membrane leakage (Dubey, 2005).

Materials and Methods

Preparation of genetic materials

Knockout *rd29a* and *rd29b* T-DNA insertion *Arabidopsis thaliana* mutants were obtained from the Arabidopsis Biological Resource Center (Ohio State University, OH). Homozygous mutants were identified by PCR-based genotyping using pROK2 T-DNA border- and gene-specific oligonucleotide primers. The pCAMBIA3301 binary vector having *GUS* gene, Kanamycin resistance gene, and BASTA® resistance gene was used to clone the promoters from *RD29A* and/or *RD29B* genes upstream of *GUS* and to replace the *35S CaMV* promoter for expression under control of the native promoters. Genomic

DNA was isolated from *Arabidopsis thaliana* wild-type plants and used to amplify *RD29A* and *RD29B* promoter regions using oligonucleotide primers with engineered EcoRI/NcoI restriction sites. *RD29A* promoter was amplified using RD29ApmtF: 5'-GAATTCAGATTTGGGGTTTTGCTTTTG-3' (Forward) and RD29ApmtR: 5'-CCATGGTCCAAAGATTTTTTTCTTTCC-3' (Reverse). *RD29B* promoter was amplified using RD29BpmtF: 5'-GAATTCCGTAATTTTCTAGATCCGTCTTGG-3' (Forward) and RD29BpmtR: 5'-CCATGGTCCAAAGCTGTGTTTTCTC-3' (Reverse).

To remove the *GUS* gene and replace it with cDNAs from *RD29A* and/or *RD29B*, pCAMBIA3301 binary vector was prepared using NcoI/PmlI restriction enzymes. The *RD29A* and *RD29B* cDNAs were amplified from reverse-transcribed RNA by PCR using oligonucleotide primers with engineered NcoI/SmaI restriction sites. These oligonucleotide primers were as follows: RD29ANcoIF: 5'-CCATGGATCAAACAGAGGAACCACC-3' (Forward) and RD29ASmaIR: 5'-CCCGGGTTAAAGCTCCTTCTGCACCGG-3' (Reverse) for *RD29A* cDNA and RD29BNcoIF: 5'-CCATGGAGTCACAGTTGACACGTCC-3' (Forward) and RD29BSmaIR: 5'-CCCGGGTCAGTTCCCAGAATCTTGAACTCC-3' (Reverse) for *RD29B* cDNA. The resulting fragments were cloned into pGEM-TEasy (Promega, Madison, WI, USA) and verified by DNA sequencing.

Four binary vectors were constructed with the *RD29A* and *RD29B* promoters upstream of the *RD29A* and *RD29B* cDNAs respectively, and two chimeric genes with the cognate gene promoters swapped. *Arabidopsis thaliana* plants were transformed with these vectors by the floral dip method using the *Agrobacterium tumefaciens* strain GV3101. Six transgenic lines were created: *RD29Apromoter::cRD29A (*or *A:A)*,

*RD29Apromoter::cRD29B (*or *A:B)*, and *RD29Bpromoter::cRD29A (*or *B:A)* were used to transform the *rd29a* knockout mutant plants. *RD29Bpromoter::cRD29B (*or *B:B)*, *RD29Apromoter::cRD29B (*or *A:B)*, and *RD29Bpromoter::cRD29A (*or *B:A)* were used to transform the *rd29b* knockout mutant plants. The transgenic *Arabidopsis* plants were rigorously tested for altered physiology under carefully controlled stress conditions. More specifically, T3 homozygous seeds from the *rd29a A:A, rd29a A:B, rd29a B:A, rd29b B:B, rd29b A:B,* and *rd29b B:A* transgenic lines were germinated in pots with soil and 4-week-old plants were subjected to either 24 hrs cold treatment at 4 °C, 4 d water deprivation, or 2 d watering with 250 mM NaCl. Gene-specific oligonucleotide primers were designed to distinguish *RD29A* and *RD29B* transcripts and determine expression levels of different transgenes by RT-PCR.

Histochemical GUS Stain

The 5' regions upstream of the translation start sites of *RD29A* and *RD29B* were cloned into pCAMBIA3301 to produce promoter::*GUS* reporter gene fusions; these plasmids were used to transform *Arabidopsis thaliana* wild-type plants to determine tissue-specific expression patterns of *RD29A* and *RD29B*. Homozygous lines were identified.

Transgenic *Arabidopsis* plants carrying *RD29Apromoter::GUS* and *RD29Bpromoter::GUS* were subjected to abiotic stresses (*i.e.*, salt and cold treatments). Seeds were placed on vertical Murashige-Skoog (MS) media plates with 2 % sucrose and 0.6 % phytagar (Gibco-BRL, Gaithesburg, MD). Plates with 14-day-old seedlings were placed overnight at 4 °C for cold treatment, or were supplied with 50 mM NaCl to

simulate salt stress. Moreover, 4-week-old plants were watered with 100 mM NaCl concentration for 4 d.

Plants were immersed in GUS stain (100 mM sodium phosphate buffer, 10 mM EDTA pH 8.0, 0.1 % Triton X-100, 2 mM Potassium Ferricyanide, 2 mM Potassium Ferrocyanide) with X-gluc (5-Bromo-4-chloro-3-indoyl Beta-D-glucuronic acid) then vacuum infiltrated for 30 minutes and incubated at 37 °C overnight. Ethanol (70 %) was used to remove chlorophyll from the samples and staining was visualized by microscopy.

Subcellular localization of RD29A and RD29B proteins

RD29A and *RD29B* cDNAs were cloned into the pEGAD binary vector to produce an in-frame fusion to GFP (Cutler et al., 2000). pEGAD with no insert, pEGAD-*RD29A* and pEGAD-*RD29B* were transformed into *Agrobacterium tumefaciens* strain GV3101 MP90 by electroporation. Transient transformation of *Arabidopsis thaliana* leaves was achieved with 'agroinfiltration' by infiltrating the strains into leaves using a syringe without a needle (Schob et al., 1997; Yang et al., 2000; Goodin et al., 2002; Stone et al., 2005). After 48 hrs, GFP was visualized with a laser scanning confocal microscope (BioRad MRC-1024ES) and analyzed using BioRad LaserSharp (v3.3) software (Bio-Rad, Hercules, CA, USA). Images shown were merged from Z-series scans.

Semiquantitative RT-PCR

Total RNA was isolated from individual 4-week-old plants grown under control conditions (Well-watered plants, grown in a growth chamber at 22 °C, 70% RH and ~130 μmol m^{-2} s^{-1} under cool white fluorescent lights supplemented with incandescent lamps with a 12 hrs photoperiod), or under the following abiotic stress conditions: 1) cold treatment of 24 hrs at 4 °C; 2) drought treatment of 4 d water deprivation; and 3) salt

treatment of 2 d watering with 250 mM NaCl, using Trizol extraction method (Invitrogen, Carlsbad, CA, USA). The quality of RNA was determined by electrophoresis using 1% agarose gel containing ethidium bromide. RNA concentration was determined spectrophotometrically. The samples were heated to 65 °C for 5 minutes and then stored on ice. Reverse transcription was performed in a 20 µL reaction with 1 µg total RNA, 0.5 µg oligo $(dT)_{18}$ primer, 40 U RNasin, 500 µM dNTPs, and 40 U M-MuLV reverse transcriptase (Fermentas, Hanover, MD, USA). The reactions were incubated at 42 °C for 1 hr, 90 °C for 2 minutes, and then stored on ice. Samples were diluted to 50 µL. PCR was performed for 30 cycles; 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 minute in a reaction containing 10 mM dNTPs, 25 mM $MgCl_2$, 10 µM oligonucleotide primers. Gene-specific oligonucleotide primers were designed to distinguish *RD29A* (newRD29AF: 5'-GGATCAAACAGAGGAACCAC-3' and newRD29AR: 5'-GCTCCTTCTGCACCGGAAC-3') and *RD29B* (RD29BF2: 5'-CGTCCTTATGGTCATGAGC-3' and RD29BR2: 5'-GCCTCATGTCCGTAAGAGG-3') transcripts by RT-PCR; individual T-DNA knockouts confirm that these primers effectively distinguish both transcripts. Actin RT-PCR was performed as a positive control for all RT-PCR reactions. The primers were as follows: ACT2-F: 5'-GTGCCAATCTACGAGGGTTTC-3' (Forward) and ACT2-R: 5'-CAATGGGACTAAAACGAAAA-3' (Reverse). Reaction products were visualized after electrophoresis on a TAE plus 1 % agarose gel containing ethidium bromide.

Arabidopsis Growth and Physiology in Response to Salt Stress

a. Root Length Sensitivity Assay

Arabidopsis thaliana wild-type (wt Col-0), both knockout mutants (*rd29a* KO and *rd29b* KO) and different transgenic lines (*rd29a A:A, rd29a A:B, rd29a B:A, rd29b B:B, rd29b A:B,* and *rd29b B:A*) were sown on agar plates (MS + 2% Sucrose + 0.6% Phytagar) supplemented with 50 mM NaCl to induce salt stress. Plates were incubated (vertically) in a growth chamber (Percival AR36L; Percival, Perry, IA) at 22 °C, 70% RH and ~130 μmol m^{-2} s^{-1} under cool white fluorescent lights supplemented with incandescent lamps with a 12 hr photoperiod. After 14 d, root lengths were measured to the nearest 10 mm. Analysis of variance was used to determine significant differences in the responses of genotypes to salt stress using the SAS statistical package (SAS Institute, Inc. 1998).

b. Physiological Responses

Physiological measurements were performed on 4-week-old wt Col-0, *rd29a* KO and *rd29b* KO plants, growing in the greenhouse at temperature of 27 °C, after daily watering with 100 mM NaCl for 4 consecutive days. Gas exchange measurements were determined on a whole plant area basis using the LI-6400XT CO_2 gas exchange analyzer fitted with a new LI-6400-17 Whole Plant Arabidopsis (WPA) chamber. The chamber is designed to measure whole plant CO_2 exchange on small rosette-type plants such as *Arabidopsis thaliana*, and is mounted with a LI-6400-18 RGB (Red, Green, Blue) Light Source designed for use with the LI-6400-17 WPA chamber (LICOR Inc., Lincoln, NE).

For gas exchange measurements using the LI-6400-17 WPA chamber, *Arabidopsis* plants were grown in 4 cm diameter containers (Cone-tainers™) fitted to the

WPA Chamber dimensions. The surface of each container's planting medium was sealed with standard pottery clay, 4-6 mm thick, placed on top of the medium adhering to the container's sidewalls to suppress any potential CO_2 flux through the planting medium. Photosynthetic measurements were determined at CO_2 concentration of 400 μmol mol^{-1}, relative humidity (RH) of 60%, air temperature (T) of 25 °C, and photosynthetically active radiation (PAR) of 500 μmol m^{-2} s^{-1}. Plants were allowed to acclimate to chamber conditions before measurements were taken.

Arabidopsis rosettes were harvested after measurements and canopy area was determined with a leaf area meter (LI3100C, LICOR Inc., Lincoln, NE).

c. Carbon Isotope Analysis

Canopies from 4-week-old wt Col-0, *rd29a* KO and *rd29b* KO plants grown under control conditions or watered with 100 mM NaCl for 4 consecutive days were collected and dried in an oven for 24 hrs at 70 °C. The leaf material was ground to a fine powder and 2-3 mg were processed through an online element analyzer (Heraeus, CHN-O Rapid) interfaced with a Finnigan Delta-S Isotope ratio mass spectrometer through a trapping box system. The carbon isotope ratio ($\delta\ ^{13}C$) of each sample was determined by relating the $^{13}C/^{12}C$ of the sample (R_{sample}) to the $^{13}C/^{12}C$ ratio of the VPDB standard ($R_{standard}$).

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d. Osmolality Determinations

Vapro® vapor pressure osmometer (WESCOR, Logan, UT) was used to measure the osmolality of 4-week-old *Arabidopsis* wild-type Col-0, *rd29a* knockout mutant and *rd29b* knockout mutant plants grown in the greenhouse under well-watered conditions or

treated with 100 mM NaCl irrigation water for 4 consecutive days. Optimol standards were used for routine osmometer calibration. Values obtained were used to calculate the osmotic potential ($\Psi\pi$ in MPa) by applying the Van't Hoff equation: $_s$, Where R the gas constant (8.314 J mol^{-1} K^{-1}), *T* temperature (K)**,** and c_s concentration of solute expressed as mol m^{-3} (mol per 10^3 kg solvent).

Results

RD29A- *and* RD29B-*promoter tissue expression and RD29A and RD29B protein subcellular localization*

The promoter::*GUS* fusions allowed us to better map tissue-specific expression patterns and stress-responsiveness of *RD29A* and *RD29B*. Expression in seedlings in response to cold and salt treatments is shown in Fig. 2.1. In 14-day-old seedlings, basal expression in control seedlings is low, but detectable (Fig. 2.1A, B). *RD29A* expression was significantly induced and ubiquitous under both cold (Fig. 2.1C) and salt (Fig.2.1E) stresses, while *RD29B* was expressed in cotyledons and roots, but not in true leaves (Fig. 2.1D, F), indicating that the *RD29B* promoter confers a distinct, and more restricted tissue expression pattern. These promoter-reporter fusion results were confirmed by RT-PCR using gene-specific oligonucleotide primers that distinguish between the *RD29A* and *RD29B* transcripts in tissues from cotyledons, true leaves, and roots (data not shown).

Because salt greatly induced the expression of both *RD29A* and *RD29B* genes, we subjected older, mature (4-week-old) *Arabidopsis* plants harboring *pRD29A::GUS* and *pRD29B::GUS* to salt stress by applying water containing 100 mM NaCl for 4 d. Our

results indicate that both promoters were highly induced in both roots and leaves in response to salt treatment (Fig. 2.2).

The RD29A and RD29B proteins were fused to the green fluorescent protein (GFP), transiently transformed into leaves, and the subcellular localization of GFP was visualized by confocal microscopy (Fig. 2.3). The positive control showed GFP expression throughout the cytoplasm and nuclei (Fig. 2.3A), whereas the GFP-RD29A (Fig. 2.3B) and the GFP-RD29B fusions (Fig. 2.3C) were targeted only to the cytoplasm, consistent with the lack of any discernible targeting sequences.

Expression of the RD29A and RD29B genes under control of the stress-responsive native promoters and chimeric gene promoter swap constructs

RD29A and *RD29B* genes were exquisitely sensitive to cold, drought and salt treatments. The *cis*-acting elements in the *RD29A* and *RD29B* promoters conferred distinct abiotic stress responsiveness. Moreover, expression of epitope-tagged *RD29A* complemented the *rd29a* mutant phenotypes, but ectopic overexpression was found to be somewhat detrimental to plant growth and development (data not shown), so the expression of the proteins was also controlled by their native promoters.

Because *RD29A* and *RD29B* are abiotic stress-responsive genes, the knockout mutants and transgenic lines were subjected to a battery of stresses to identify quantifiable phenotypes (Fig. 2.4). Semiquantitative RT-PCR with gene-specific oligonucleotide primers effectively distinguished between the closely related *RD29A* and *RD29B* transcripts. Under control conditions, expression of both genes was nearly undetectable in wild-type Col-0 or the *rd29a* and *rd29b* T-DNA knockout mutant plants. The *rd29a* and *rd29b* knockout plants were verified to be true null mutants, showing no

expression in response to cold, drought or salt treatments. Transgenes showed variable levels of expression under control conditions (Fig. 2.4A). When subjected to abiotic stresses, our results indicated that cold highly induced the *RD29A* gene (Fig. 2.4B). Transformation of the *rd29a* knockout mutant plants with the *RD29Apromoter::cRD29A* (AA) construct fully complemented, and the transcript levels were similar to those of wild-type plants (Fig. 2.4B). The *RD29A* promoter was more responsive to cold than the *RD29B* promoter; the *RD29Apromoter* confers high expression of *cRD29B* (AB) in the knockout mutant plants (Fig. 2.4B). After 4 d of water deprivation, *RD29A* was highly expressed, whereas *RD29B* showed a less significant response to drought (Fig. 2.4C). After salt treatment, high expression of both *RD29A* and *RD29B* genes was noticed. The promoters of both genes were highly induced by salt stress (Fig. 2.4D) in both the wild-type and transgenic plants. Interestingly, in response to drought, the *RD29B* was extremely highly induced by drought in the *rd29a* mutants and vice versa, suggesting some sort of compensatory effect (Fig. 2.4C). This was not observed for both the cold and salt treatments, and appears to be unique to the drought stress.

Morphological and physiological responses of wild-type, knockout and transgenic Arabidopsis plants to salt stress

Primary root lengths in wild type (wt Col-0) did not statistically differ from knockout mutants or transgenic lines under control conditions. Salt stress, as expected, resulted in a significant decline *($p<0.001$)* in root length in wt Col-0, as well as all transgenic lines relative to control. This decline however was significantly less pronounced in *rd29a* and *rd29b* knockout mutants, which exhibited significantly greater root length under salt treatment ($p<0.001$) compared to wt Col-0 and the different

transgenic lines produced in both knockout backgrounds (Fig. 2.5). Therefore, both the *rd29a* and *rd29b* knockout mutants are somewhat resistant to salt-stress induced inhibition of root elongation, and all of the "complementing" transgenes can restore sensitivity (Fig. 2.5).

Given the unexpected, yet intriguing, resistance to salt stress-induced root growth inhibition in *rd29a* and *rd29b* knockout mutants, we further investigated the physiological responses of these mutants to salt stress. Gas exchange measurements indicated that photosynthesis (A) and transpiration rates (E) did not differ between wt Col-0 and *rd29a* and *rd29b* knockout mutants under control conditions. Salt stress caused a significant decrease in A and E ($p<0.001$) in wild-type plants, but the *rd29a* and *rd29b* knockout mutants maintained ~50% higher photosynthetic rates (A) compared to their wild-type counterpart (Fig. 2.6A). However, E did not significantly differ between lines under salt stress (Fig. 2.6A), resulting in a significant improvement in instantaneous water use efficiency (WUE, which is a measure of the amount of photosynthate produced per unit of water consumed) in *rd29a* and *rd29b* knockout mutants relative to wt Col-0 and control ($p<0.001$, Fig. 2.6C).

Carbon isotope ratio ($\delta\ ^{13}C$), which is a measure of integrated WUE over the life of the tissue, was significantly ($p<0.001$) less negative (higher) under salt treatment than in control indicating significant enrichment (*i.e.,* less discrimination) of ^{13}C in tissues under stress and improvement of WUE. No significant difference in the carbon isotope ratio was observed for these genotypes (Fig. 2.7A). Osmotic potential, which accounts for the majority of dissolved solute particles in a solution, became significantly more negative under salt stress, with solute concentration increasing 2.6-fold under salt stress.

Various genotypes did not significantly differ among each other within a treatment (Fig. 2.7B).

Discussion

The *RD29A* and *RD29B* genes are stress-inducible and not expressed at significant levels under what is considered optimal or normal plant conditions. Therefore, their upstream promoter sequences could be used to confer distinct abiotic stress resistance in some plants by driving expression of transcription factors that control stress "regulons". These genes encoding closely related proteins of unknown function appear to be limited to the *Brassicaceae* family. Cold, drought, or salt stress can affect the *RD29A* and *RD29B* expression through ABA-dependent or ABA-independent signal transduction pathways; these observed expression patterns might be due to the presence of ABRE and DRE motifs in their promoters. The *RD29A* promoter contains several DREs and one ABRE and the *RD29B* promoter has several ABREs and one DRE (Zhang et al., 2004; Sakuma et al., 2006; Sakuma et al., 2006). Exogenous application of ABA significantly induces the expression of both *RD29A* and *RD29B* within 3 hrs by 200-fold and 40-fold respectively (Zimmermann et al., 2004; Toufighi et al., 2005; Zimmermann et al., 2005; Nemhauser et al., 2006).

An epitope-tagged version of *RD29A* (NTAPi-RD29A) under control of the *35S CaMV* promoter, introduced into the wild-type Col-0 and the *rd29a* knockout mutant backgrounds, revealed that the *rd29a* knockout mutant phenotypes were effectively complemented (data not shown). The *RD29A* promoter appears to be induced by cold, drought, and salt, and the *RD29B* promoter is less stress-responsive. Moreover,

transformation of *rd29a* and *rd29b* knockout mutant plants using chimeric gene constructs with the native promoters swapped showed that the promoter of *RD29A* was responsive to cold, drought, and salt stresses, while the promoter of *RD29B* was highly responsive to salt stress.

The proteins encoded by the *RD29A* and *RD29B* genes remain uncharacterized. The extent and mechanism of (presumed) protection conferred by these proteins remains poorly understood. Their products have resemblance to late embryogenesis abundant (LEA) proteins and hence are called LEA-like proteins (Yamaguchi-Shinozaki and Shinozaki, 1993; Kasuga et al., 1999; Kaur and Gupta, 2005). Genes encoding LEA proteins have also been used to improve abiotic stress tolerance in transgenic plants (Shinozaki and Yamaguchi-Shinozaki, 2007). Extremely hydrophilic LEA proteins have been proposed to act as chaperones, surfactants, or ion chelators (Smirnoff and Bryant, 1999), but further studies are necessary to understand their function under stress.

Under controlled conditions (absence of salt stress), measured physiological parameters were within the ranges reported for *Arabidopsis thaliana* (LICOR Inc., Lincoln, NE). Growth and photosynthetic properties declined significantly under salt stress. Salinity is known to have very strong impacts on photosynthetic properties, due to ionic, osmotic and oxidative stresses (Mudrick et al., 2003). The increase of ionic and/or osmotic stress, leads to a decline in stomatal conductance and internal CO_2 (C_i), and a decrease in ribulose-bisphosphate (RuBP) regeneration, resulting in oxidative stress and declines in photochemistry and carboxylation efficiency, and subsequently shoot and root growth. In this study, the observed reductions in A and E due to increased stomatal resistance, and the resulting increases in WUE, tissue ^{13}C enrichment, and tissue solute

concentration (more negative Ψ_π) are in agreement with what has been reported for other species under salt stress (*e.g.*, Clark et al., 1999; Mudrick et al., 2003; Koyro, 2006), and have been suggested to act as mechanisms by which many plant species cope and resist excessive salt. This resistance strategy is aimed to reduce both salt loading into the leaves and the subsequent injuries by keeping salts at subtoxic levels (Koyro, 2006).

Contrary to our original hypothesis, the *rd29a* and *rd29b* knockout mutant lines maintained greater root growth, A, and WUE under salt stress relative to control. This was unexpected as the *RD29A* and *RD29B* genes were both highly induced by salt stress. Moreover, the resistance to salt stress-induced root growth inhibition was effectively complemented (*i.e.*, sensitivity was restored) by all of the transgenes, regardless of the promoter:cDNA combinations, suggesting a possible redundancy in function and consistent with the fact that *RD29A* and *RD29B* genes encode very closely related proteins. Unfortunately, we have been unable to generate a double *rd29a/rd29b* mutant to directly address this possibility. Whereas this negative result might indicate that a double mutant would be lethal, we are unlikely to ever recover a recombination event between the two genes because the genes are tandemly arrayed in the genome (linked). RNA-interference approaches may aid in answering this question, but is limited by the inability to effectively silence genes. Because loss-of-function results in resistance to stress (at least salt stress as assayed here), our results with the single mutants suggest that the RD29A and RD29B proteins are unlikely to serve as protective molecules (as predicted from their transcriptional responsiveness), but perhaps may function as warning signals for abiotic stress responses. Another unexpected outcome of this work is that we observed essentially wild-type induction of *RD29A* or *RD29B* transcripts in the "sister

gene" knockout mutants in response to cold and salt stresses, but the "sister" genes were massively induced in response to drought stress. These data suggest a compensatory mechanism is in place, at least in the case of drought. Further studies will be needed to better understand the common and distinct roles of the *RD29A* and *RD29B* genes in abiotic stress responses.

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Figures

Figure 2.1 Representative tissue expression patterns of the *RD29A* and *RD29B* genes in seedlings in response to cold and salt stresses**.**

Histochemical staining for β-glucuronidase activity performed on homozygous transgenic 14-days-old seedlings harboring *pRD29A::GUS* (Panels A, C, and E) and *pRD29B::GUS* (Panels B, D, and F). Seedlings in panels A and B were grown on plates with 12 hrs photoperiod control conditions, seedlings in panels C and D were subjected to 24 hrs cold treatment at 4 °C, seedlings in panels E and F were sown on MS plates supplied with 50 mM NaCl before staining with X-Gluc.

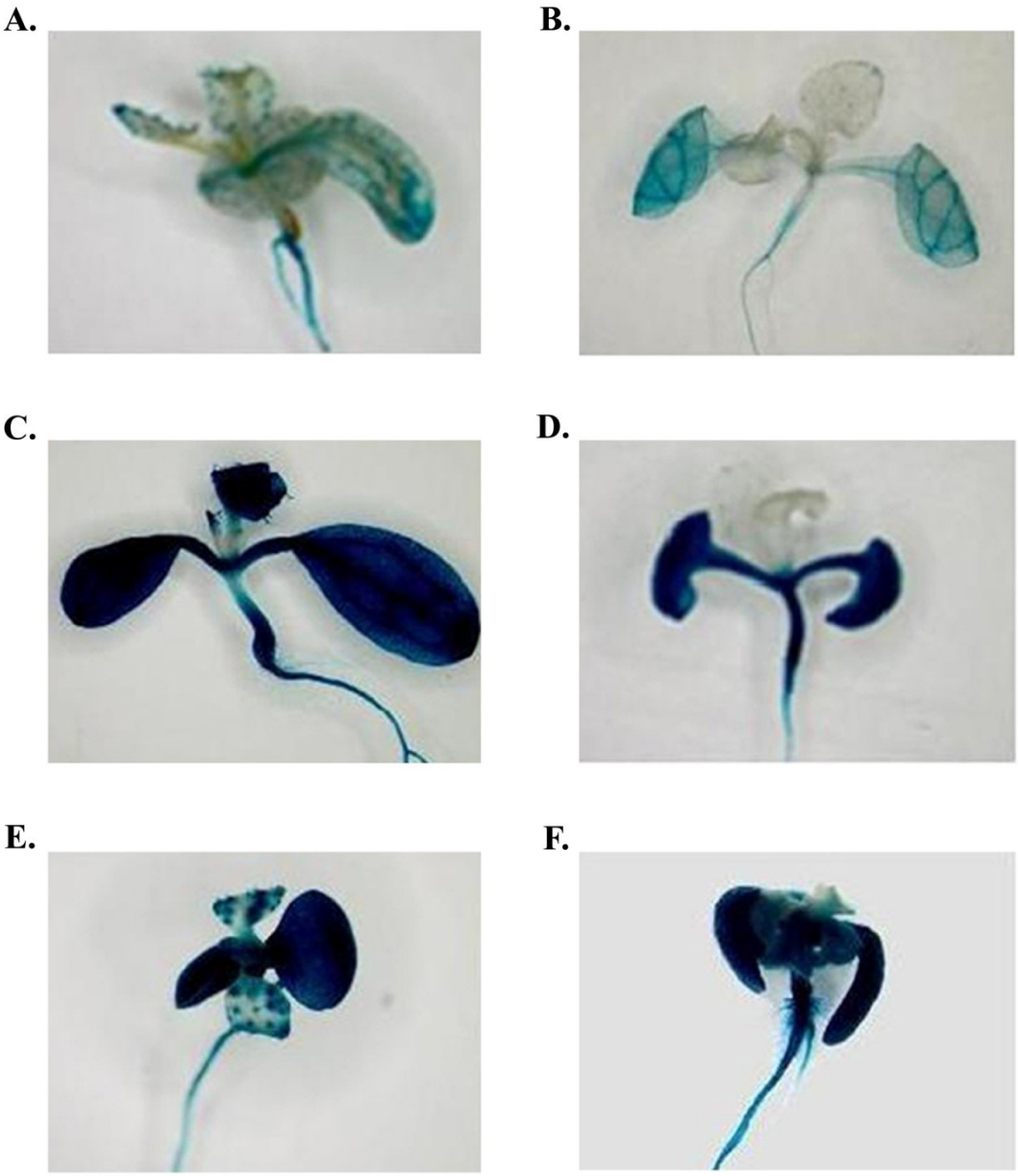

A.
B.
C.
D.
E.
F.

Figure 2.2 Representative tissue expression patterns of the *RD29A* and *RD29B* genes in mature plants in response to cold and salt stresses.

Histochemical staining for β-glucuronidase activity performed on homozygous transgenic 4-week-old plants harboring *pRD29A::GUS* (Panels A, and C) and *pRD29B::GUS* (Panels B, and D). Plants in panels A and B were grown on soil and placed in a 22 °C growth chamber with a 12 hrs photoperiod control conditions; plants in panels C and D were grown on soil watered with 100 mM NaCl concentration for 4 d before staining with X-Gluc.

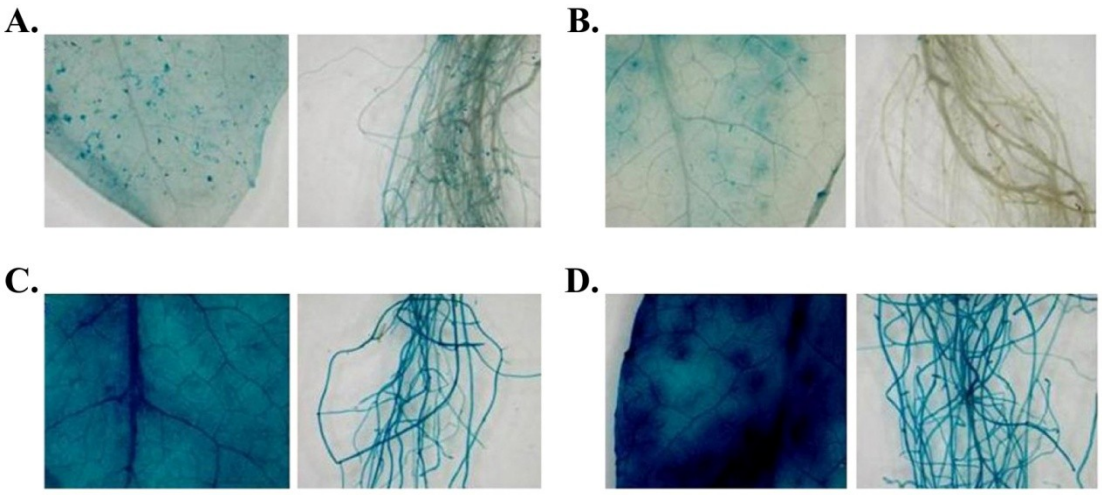


Figure 2.3 GFP-RD29A/B fusion proteins are subcellularly localized to the cytoplasm. (A) Transient transformation with pEGAD (positive control) showed expression of *GFP* throughout the cell (cytoplasm and nucleus). (B) and (C) Transient transformations with *pEGAD-RD29A/B* showed RD29A and RD29B localized to the cytoplasm. Images were obtained by confocal laser scanning microscopy (bar= 50 µm).

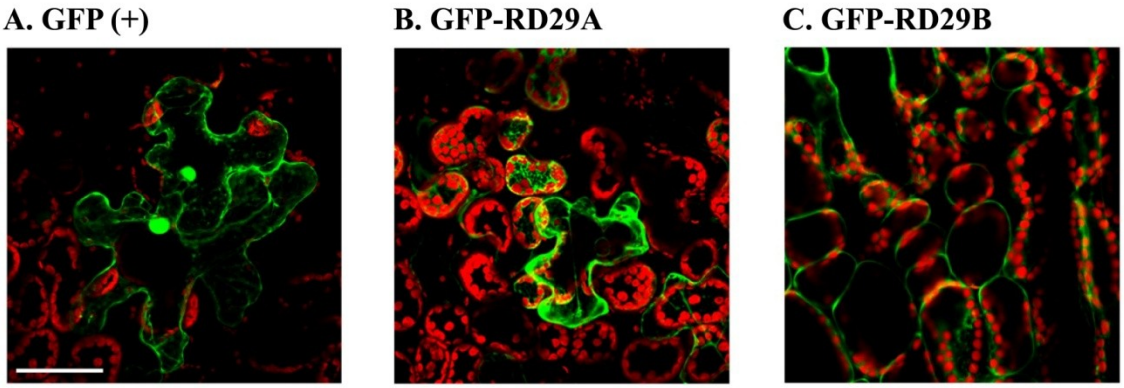


Figure 2.4 *RD29A* and *RD29B* gene expression under control of various combinations of promoters and cDNAs in *rd29a* and *rd29b* mutants.

Steady-state *RD29A* and *RD29B* transcript levels were determined by semiquantitative RT-PCR using oligonucleotide primers that distinguish between the two closely related genes in tissues derived from 4-week-old plants subjected to different abiotic stresses. Genotypes are indicated for wild-type *Arabidopsis thaliana* plants (Col-0), *rd29a/b* knockouts, *rd29a* knockout transformed with AA or AB or BA transgenes and *rd29b* knockout transformed with BB or AB or BA transgenes. AA, BB refer to the transgenes with cDNAs under control of their native promoters, whereas AB and BA refer to transgenes when the native promoters were swapped. Actin was used as an RNA loading control. (A) Control conditions: Well-watered plants, grown in a 22 °C growth chamber with 12 hrs photoperiod. (B) Cold treatment: 24 hrs at 4 °C. (C) Drought treatment: 4 d water deprivation. (D) Salt treatment: 2 d watering with 250 mM NaCl.

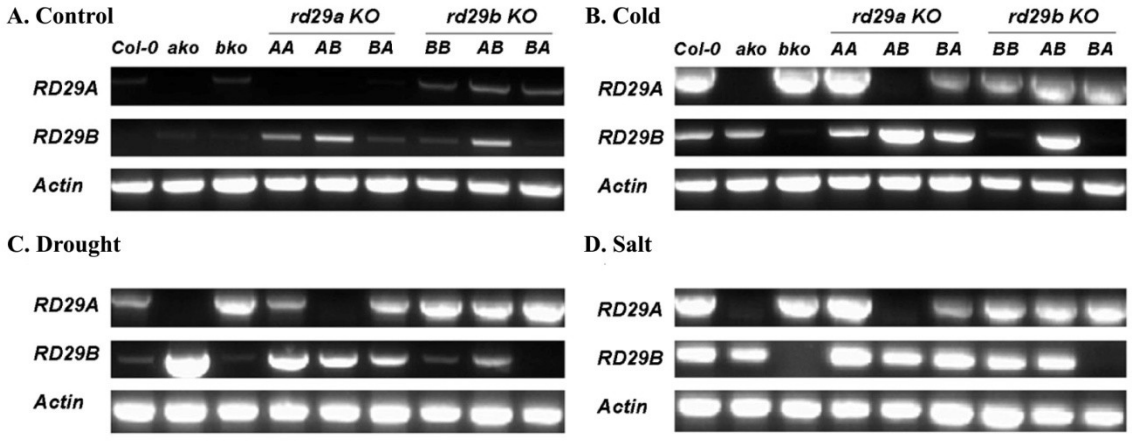
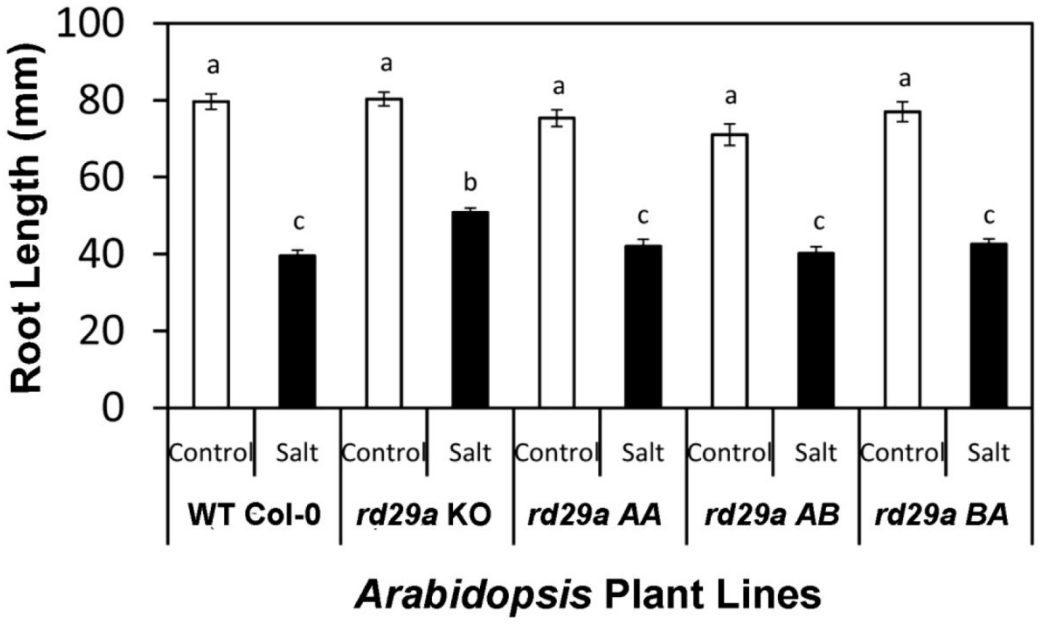


Figure 2.5 Physiological effects of salt stress on root length in *rd29a* and *rd29b* mutants and transgenic plants.

Root length (mm) measured for 14-day-old seedlings growing on MS-agar plates (white bars) or or supplemented with 50 mM NaCl (black bars). (A) *rd29a* background; (B) *rd29b* background. Different letters indicate significant differences among treatments or genotypes ($P<0.001$).

A. *rd29a* Background



B. *rd29b* Background

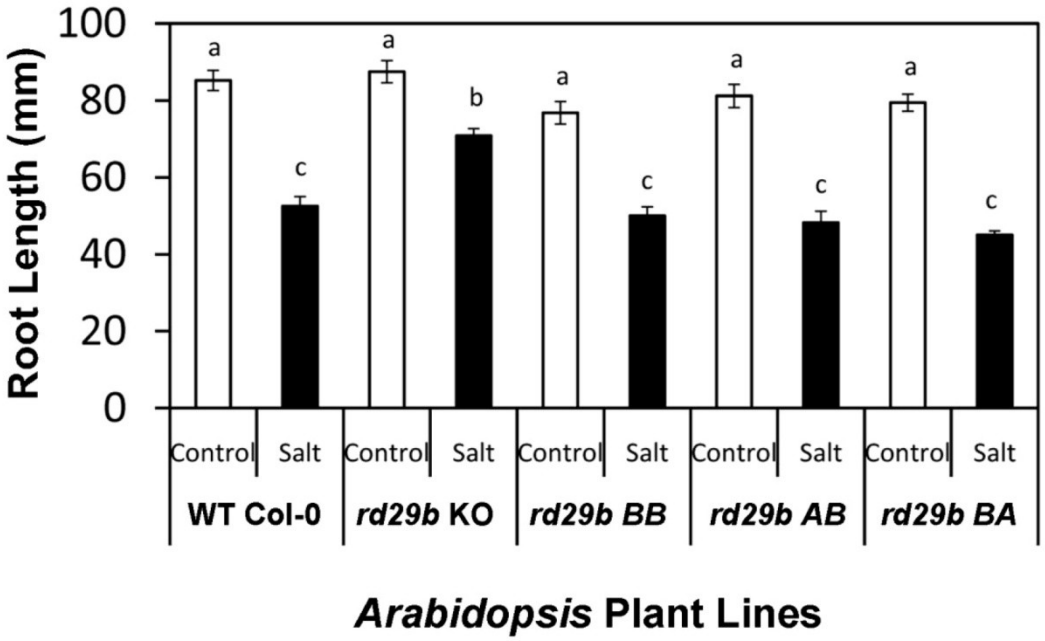


Figure 2.6 Physiological responses of wild-type and *rd29a* and *rd29b* mutants in response to salt stress.

Photosynthesis (A), transpiration rates (B) and water use efficiency (C) of 4-week-old wild-type (wt Col-0), *rd29a* knockout mutant (*rd29a* KO), and *rd29b* knockout mutant (*rd29b* bKO). Plants were grown in the greenhouse under well-watered conditions (white bars) or treated with 100 mM NaCl irrigation water for 4 consecutive days (black bars). Different letters within each graph indicate significant differences among treatments or genotypes ($P<0.001$).

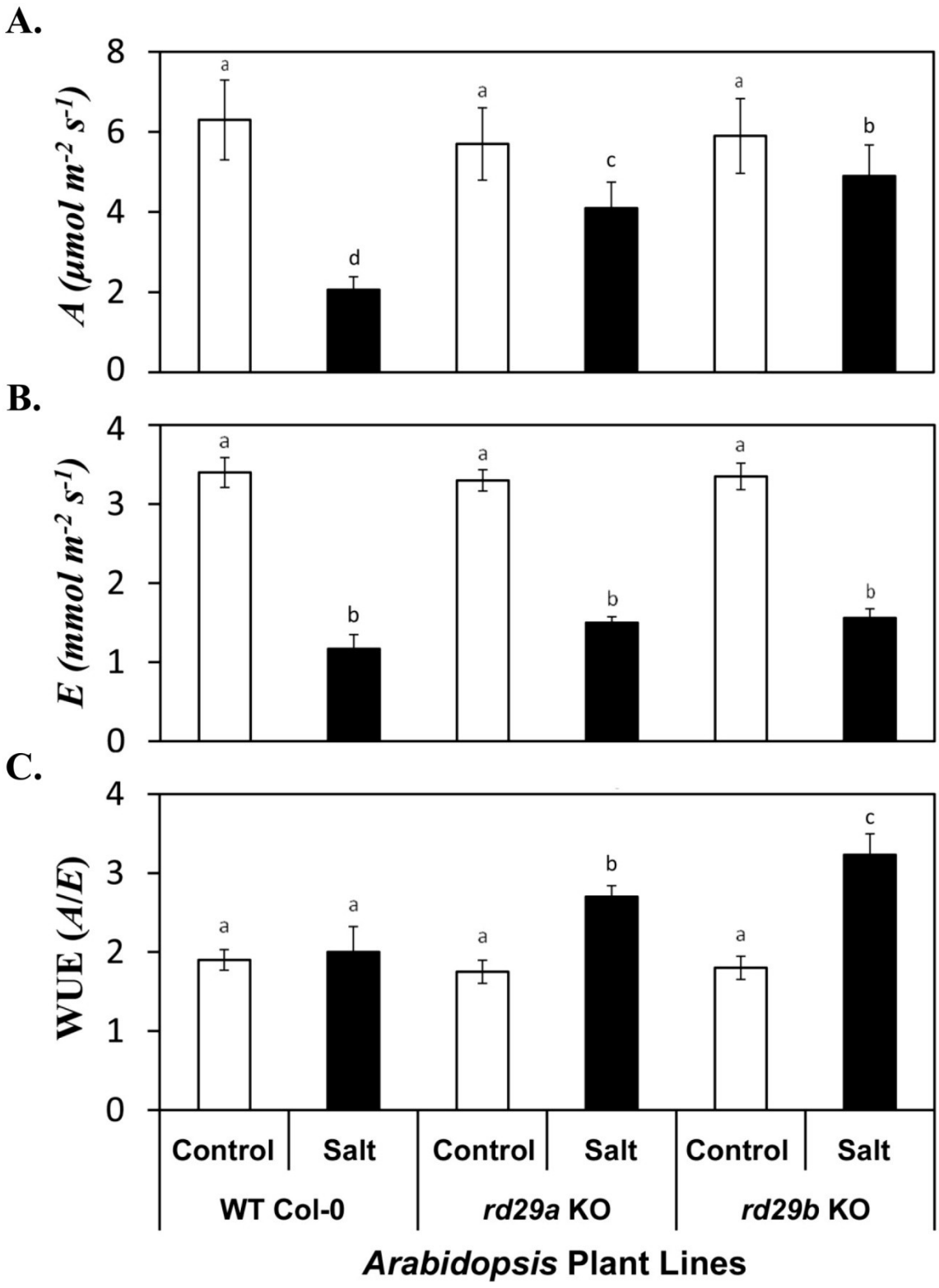
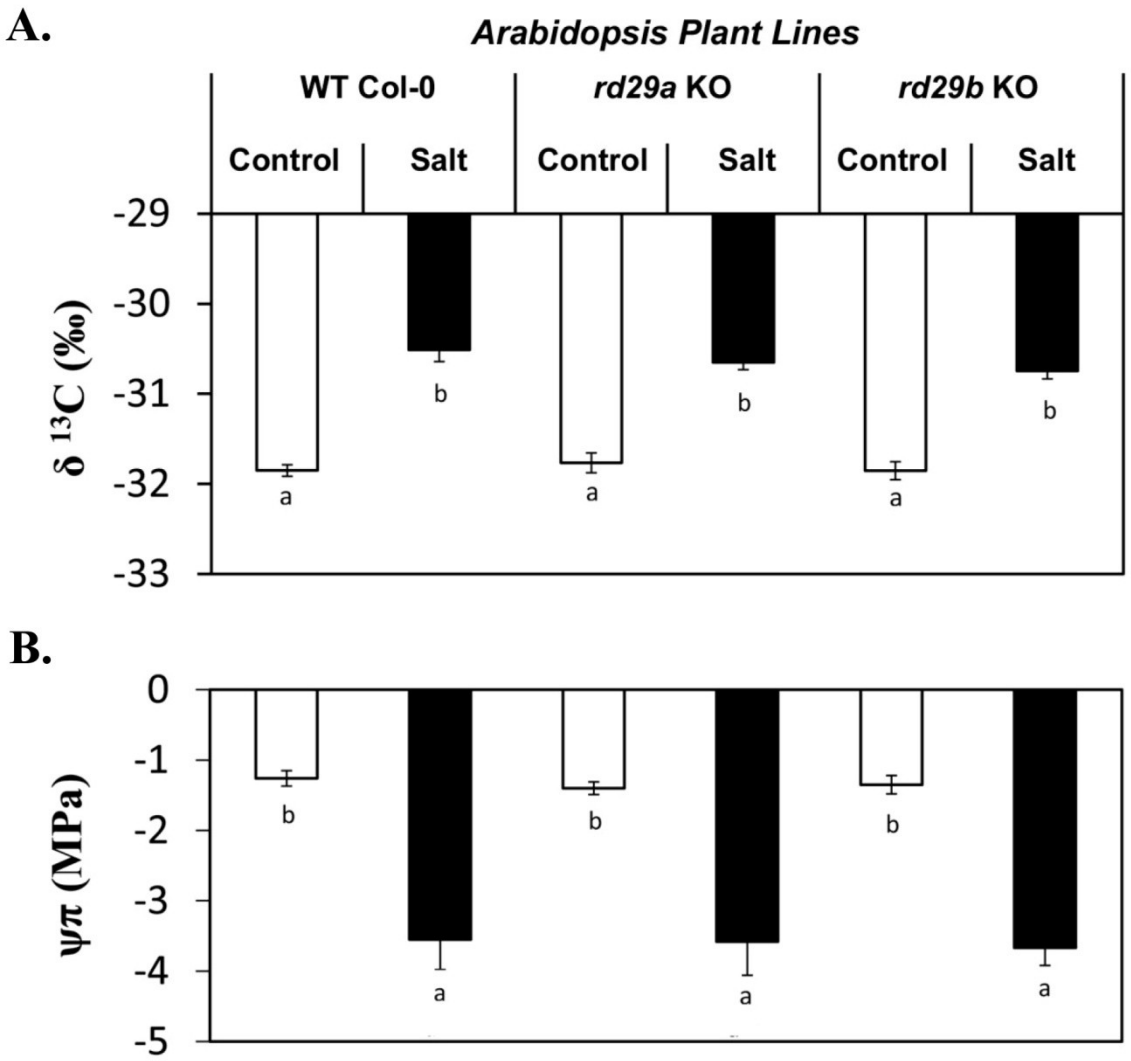

A.
A (μmol m-2 s-1)
8
6
4
2
0
a
d
a
c
a
b
B.
E (mmol m-2 s-1)
4
3
2
1
0
a
b
a
b
a
b
C.
WUE (A/E)
4
3
2
1
0
a
a
a
b
a
c
Control
Salt
Control
Salt
Control
Salt
WT Col-0
rd29a KO
rd29b KO
Arabidopsis Plant Lines

Figure 2.7 Physiological responses of wild-type and *rd29a* and *rd29b* mutants in response to salt stress.

Carbon isotope ratio (A) and osmotic potential (B) determined using the canopies of 4-week-old wild-type (wt Col-0), *rd29a* knockout mutant (*rd29a* KO), and *rd29b* knockout mutant (*rd29b* bKO) plants. Plants were grown in the greenhouse under well-watered conditions (white bars) or treated with 100 mM NaCl irrigation water for 4 consecutive days (black bars). Different letters within each graph indicate significant differences among treatments or genotypes ($P<0.001$).



CHAPTER 3 - METABOLIC AND GENE EXPRESSION CHANGES TRIGGERED BY NITROGEN DEPRIVATION IN THE PHOTOAUTOTROPHICALLY GROWN MICROALGAE *Chlamydomonas reinhardtii* AND *Coccomyxa* sp. C-169

Abstract

Microalgae are emerging as suitable feedstocks for renewable biofuel production. Characterizing the metabolic pathways involved in the biosynthesis of energy-rich compounds, such as lipids and carbohydrates, and the environmental factors influencing their accumulation is necessary to realize the full potential of these organisms as energy resources. The model green alga *C. reinhardtii* has already been shown to accumulate significant amounts of triacylglycerols (TAGs) under nitrogen starvation or salt stress in medium containing acetate. However, since cultivation of microalgae for biofuel production may need to rely on sunlight as the main source of energy for biomass synthesis, we examined metabolic and gene expression changes occurring in *Chlamydomonas* and *Coccomyxa* subjected to nitrogen deprivation under strictly photoautotrophic conditions. Interestingly, nutrient depletion triggered a similar pattern of early synthesis of starch followed by substantial TAG accumulation in both of these fairly divergent green microalgae. We also observed a marked decrease in chlorophyll and soluble protein contents, including reduction in ribosomal polypeptides and some key enzymes for CO_2 assimilation like ribulose-1,5-bisphosphate carboxylase/oxygenase. Our results suggest that turnover of nitrogen-rich compounds such as proteins may provide carbon skeletons for TAG biosynthesis in the nutrient deprived cells. In *Chlamydomonas*, several genes coding for diacylglycerol:acyl-CoA acyltransferases, catalyzing the

acylation of diacylglycerol to TAG, displayed increased transcript abundance under nitrogen depletion but, counterintuitively, genes encoding enzymes for *de novo* fatty acid synthesis, such as 3-ketoacyl-ACP synthase I, were down-regulated. Understanding the interdependence of these anabolic and catabolic processes and their regulation may allow the engineering of algal strains with improved capacity to convert their biomass into useful biofuel precursors.

Keywords: chlorophytes, starch, triacylglycerols, diacylglycerol:acyl-CoA acyltransferase, 3-ketoacyl-ACP synthase, biomass, biofuels.

Introduction

Algae are a diverse group of eukaryotic organisms with important roles in marine, freshwater and even terrestrial ecosystems. For instance, 30 to 50% of the planetary net photosynthetic productivity (the difference between autotrophic gross photosynthesis and respiration) is of marine origin and dependent on phytoplankton biomass (Field et al., 1998; Boyce et al., 2010). Recently, the great potential of algal species as feedstocks for renewable biofuel production has also gained recognition (Hu et al., 2008; Scott et al., 2010; Wijffels and Barbosa, 2010). Unicellular microalgae are capable of harnessing sunlight and CO_2 to produce energy-rich chemical compounds, such as lipids and carbohydrates, which can be converted into fuels (Hu et al., 2008; Rodolfi et al., 2009; Wijffels and Barbosa, 2010). However, the commercial production of algal biofuels is currently hindered by limitations in the biological productivity of strains, culture systems

and harvesting/extraction processes (Sheehan et al., 1998; Hu et al., 2008; Scott et al., 2010; Wijffels and Barbosa, 2010). As recently proposed, a multidisciplinary approach, including advances in basic biology and metabolic engineering of algal strains as well as in culture systems, bioprocessing and integrated biorefinery design, may be required to realize the full potential of microalgae as sustainable biofuel sources (Hu et al., 2008; Scott et al., 2010; Wijffels and Barbosa, 2010).

Desirable strain characteristics include rapid growth rate, high product content, tolerance to variable environmental conditions, resistance to predators and viruses, and ease of harvest and extraction (Griffiths and Harrison, 2009; Rodolfi et al., 2009; Radakovits et al., 2010). An additional algal feature for biodiesel production is the suitability of the lipid composition, with triacylglycerols (TAGs) being the preferred substrate (Schenk et al., 2008; Radakovits et al., 2010). Algae synthesize, under optimal growth conditions, fatty acids predominantly for esterification into glycerol-based membrane lipids (Guschina and Harwood, 2006; Hu et al., 2008; Khozin-Goldberg and Cohen, 2011). However, factors such as temperature, irradiance and nutrient availability affect both lipid composition and lipid content in many algal species (Guschina and Harwood, 2006; Hu et al., 2008; Khozin-Goldberg and Cohen, 2011; Siaut et al., 2011). Upon certain environmental stresses (particularly nutrient shortage), various algae accumulate energy-rich storage compounds such as starch and TAGs (Guschina and Harwood, 2006; Hu et al., 2008; Rodolfi et al., 2009; Wang et al., 2009; Dean et al., 2010; Li et al., 2010; Moellering and Benning, 2010; Work et al., 2010; Fan et al., 2011; Siaut et al., 2011). Fatty acids are the common precursors for the formation of both membrane lipids (required for growth) and TAGs (involved in energy storage and fatty

acid homeostasis), but it remains to be elucidated how algal cells coordinate the distribution of these precursors to distinct destinations in response to environmental stimuli (Sheehan et al., 1998; Guschina and Harwood, 2006; Hu et al., 2008; Radakovits et al., 2010). Interestingly, biomass productivity and lipid content appear to be inversely correlated in many algae (Sheehan et al., 1998; Hu et al., 2008; Rodolfi et al., 2009) and nutrient limitation stimulates TAG accumulation but at the expense of growth (Rodolfi et al., 2009; Li et al., 2011).

Given the outlined constraints, improving algal strain performance will require a greater understanding of carbon allocation between biosynthetic pathways and of the regulatory mechanisms controlling this distribution, particularly in response to environmental stresses. This need is also emphasized by the limited success in increasing total oil content in higher plants and algae by the direct engineering of single lipid biosynthesis components (Dunahay et al., 1996; Durret et al., 2008; Li et al., 2010; Radakovits et al., 2010; Work et al., 2010). The unicellular green alga *Chlamydomonas reinhardtii* has emerged as a model system for studying algal physiology, photosynthesis, metabolism, and flagellar structure and function (Harris, 2001; Merchant et al., 2007). The nuclear, plastid and mitochondrial genomes have been sequenced and a set of genomic, molecular and genetic tools is also available for this organism (Harris, 2001; Grossman et al., 2007; Merchant et al., 2007). *Chlamydomonas* has been shown to accumulate significant amounts of TAGs under nitrogen starvation or salt stress (Wang et al., 2009; Dean et al., 2010; Li et al., 2010; Moellering and Benning, 2010; Work et al., 2010; Fan et al., 2011; James et al., 2011; Siaut et al., 2011). However, most experiments characterizing storage lipid synthesis in *C. reinhardtii* have been carried out under

photoheterotrophic conditions, in acetate-containing medium (Wang et al., 2009; Li et al., 2010; Moellering and Benning, 2010; Work et al., 2010; Fan et al., 2011; James et al., 2011; Siaut et al., 2011).

Large-scale cultivation of microalgae for biofuel production may need to be based on sunlight, captured by photosynthesis, as the main source of energy (Hu et al., 2008; Griffiths and Harrison, 2009; Rodolfi et al., 2009; Scott et al., 2010; Wijffels and Barbosa, 2010). Thus, we examined metabolic and gene expression changes occurring in *Chlamydomonas* subject to nitrogen deprivation under strictly photoautotrophic conditions. We have also analyzed TAG and starch synthesis, under analogous environmental conditions, in another alga with sequenced nuclear, plastid and mitochondrial genomes, *Coccomyxa* sp. C-169 (Smith et al., 2011). This microalga belongs to the class Trebouxiophyceae, within the division Chlorophyta (Palmqvist et al., 1997; Zoller and Lutzoni, 2003), and it is very divergent in phylogeny and habitat from *C. reinhardtii*. Interestingly, nitrogen depletion triggered a similar pattern of early synthesis of starch followed by significant TAG accumulation in these fairly divergent green microalgae. Our results also suggest that turnover of nitrogen-rich compounds in the nutrient starved cells may provide carbon skeletons for TAG biosynthesis. Understanding the interdependence of these anabolic and catabolic pathways and their regulation may be key for the metabolic engineering of algal strains with improved biofuel productivity.

Materials and Methods

Strains and culture conditions

Chlamydomonas reinhardtii CC-125 and *Coccomyxa* sp. C-169 were used in all the reported experiments. Unless stated otherwise, cultures were incubated under continuous illumination (200 μmol m^{-2} s^{-1} photosynthetically active radiation) on an orbital shaker (190 rpm) at 25°C and ambient levels of CO_2. Cells were initially grown photoautotrophically to the middle of the logarithmic phase in nitrogen replete High Salt (HS) medium (Sueoka, 1960), in the case of *Chlamydomonas*, or Bold's Basal Medium (BBM) (Bold, 1949), in the case of *Coccomyxa*. These pre-cultured cells were collected by centrifugation and resuspended at a density of 0.5-1.0 x 10^6 cells mL^{-1} in regular HS or BBM media or in the same media lacking nitrogen (HS-N or BBM-N). Samples for analysis were taken immediately after resuspension (0 d) and at the times indicated in the figures and tables. Culture growth was monitored by counting cells with a hemocytometer (Harris, 1989).

Fluorescence microscopy

To assess the effect of nitrogen deprivation on nonpolar lipid accumulation, cells were stained with the lipophilic fluorophore Nile Red (Greenspan et al., 1985) as previously described (Chen et al., 2009). Images were acquired with a laser scanning confocal microscope (Olympus Fluoview 500), using an 100x oil immersion lens, and analyzed with the Fluoview (v4.3) software. Laser excitation was at an emission wavelength of 443 nm and Nile Red fluorescence was detected between 560 nm and 590 nm using band-pass filtering. In the presence of neutral lipids, Nile Red emits a yellow-

gold fluorescence (λ_{max}= 580 nm) (Greenspan et al., 1985), which is shown as artificial red coloring in Figs. 3.1B and 3.5A.

Lipid analyses

Total lipids were extracted with a Bligh and Dyer (1959) procedure, transmethylated and quantified as fatty acid methyl esters (FAMEs) by gas chromatography with flame ionization detection (GC-FID) as described previously (Cahoon et al., 2006). Briefly, ~1.5 x 10^8 cells were collected by centrifugation in a 13 x 100 mm glass test tube with Teflon-lined screw cap. After removal of culture media by aspiration, 300 μg of triheptadecanoin (Nu-Chek Prep) was added as an internal standard from a stock solution at a concentration of 10 mg mL^{-1} in toluene. Lipids were then extracted from the cell pellet using a modification of the method described by Bligh and Dyer (1959). Three mL of 2:1 v/v methanol:chloroform containing 0.01% (w/v) butylated hydroxytoluene were added to the cell pellet and incubated at 25°C for 30 minutes. After addition of 1 mL of chloroform and 1.8 mL of water, the tube was shaken vigorously and the content partitioned into two phases by centrifugation at 1,000 x *g*. The upper phase was discarded and the lower organic phase, containing the extracted lipids, was transferred to a new glass tube. Extracted lipids were dried under a stream of nitrogen and resuspended in 0.5 mL of 6:1 v/v chloroform:methanol. One fourth of this extract was used for measuring total fatty acid content and the remainder was used for measuring TAGs (see below). For analysis of the content and composition of fatty acids, 125 μL of the lipid extract was transferred to another glass screw cap test tube, dried under nitrogen, and resuspended in 250 μL of toluene and 1 mL of 2.5% (v/v) sulfuric acid in methanol. The tube was capped under nitrogen and heated at 90°C for 30 min. Upon cooling, 0.5

mL of water and 0.7 mL of heptane were added. The tube was shaken vigorously, and the contents were separated into two phases by centrifugation. The upper heptane layer containing FAMEs was analyzed using an Agilent 7890 gas chromatograph (Agilent Technologies) fitted with an Agilent INNOWax column (0.25 mm inner diameter x 30 cm length). The oven temperature was programmed from 185°C (1 min hold) to 235°C (2.5 min hold) at a rate of 7°C/min with hydrogen as the carrier gas. FAMEs levels were quantified relative to the methyl heptadecanoate from the internal standard. Different fatty acid methyl esters were identified by mobility relative to standards as well as by gas chromatography-mass spectrometry using an Agilent 7890A gas chromatograph interfaced with an Agilent 5975C mass selective detector. Chromatography conditions in the latter case were the same as described above, except for the use of helium as the carrier gas.

For analysis of TAGs, the remainder of the total lipid extract was dried under nitrogen and resuspended in 100 µL of 6:1 (v/v) chloroform:methanol. This extract was applied to a silica 60 thin layer chromatography plate (Sigma-Aldrich) and neutral lipids were resolved using a solvent system of 70:30:1 (v/v/v) heptane:ethyl ether:acetic acid. The TAG band was identified by co-migration with a TAG standard, stained lightly with iodine vapors, in an adjacent lane. The TAG fraction was then recovered from the plate and lipids resuspended in 0.4 mL of toluene and 1.5 mL of 2.5% (v/v) sulfuric acid in methanol. Fatty acid methyl esters were prepared, extracted, and analyzed by gas chromatography as described above.

Double bond positions of unsaturated fatty acids were confirmed by GC-MS of derivatives generated from fatty acid methyl esters. Pyrrolidine derivatives were prepared

and analyzed for double bond positions of polyunsaturated fatty acids (Andersson et al., 1975), and dimethyl disulfide derivatives were prepared and analyzed for double bond positions of monounsaturated fatty acids (Cahoon et al., 1994). Analyses of fatty acid derivatives was conducted with an Agilent 7890A gas chromatograph interfaced with an Agilent 5975C mass selective detector fitted with a DB-1MS column (Agilent; 30 m length X 0.25 mm inner diameter X 0.25 μM film thickness). The GC oven was programmed from 185°C (1 min hold) at 10°C/min to 320°C (10 min hold).

Starch assays

Starch measurements were performed using an ethanol-washed chlorophyll-free cell pellet (Ball et al., 1990). In brief, ~2.0 x 10^7 cells were harvested by centrifugation, resuspended in an ethanol solution for chlorophyll extraction and then centrifuged again. Pellets were resuspended in distilled water and boiled for 10 min to solubilize the starch. In the case of *Coccomyxa*, resuspended pellets were autoclaved for 15 min at 120°C for starch solubilization. Total starch was quantified using a commercial kit (Starch Assay Kit SA-20; Sigma-Aldrich), based on amyloglucosidase digestion to convert starch to glucose, according to the manufacturer's instructions.

Chlorophyll measurements

Chlorophyll content was determined using ethanol extraction (Arnon, 1949). One milliliter of culture, at a concentration of 1.0 x 10^7 cells mL^{-1}, was centrifuged and the pellet resuspended in 96% ethanol and vortexed to extract pigments. Cellular debris was pelleted by centrifugation and chlorophyll *a* and *b* levels were determined spectrophotometrically, in the supernatant, by measuring optical absorbance at 645 nm

and 663 nm. Calculations of total chlorophyll ($\mu g\ mL^{-1}$) were performed as previously described (Arnon, 1949; Harris, 1989).

Protein determination

The concentration of solubilized proteins extracted from ~5.0 x 10^6 *Chlamydomonas* cells was determined using the Bio-Rad Protein Assay, by measuring absorbance at 595 nm with a microplate reader. Briefly, cells were sonicated for 20s (3x) in 250 μL of lysis buffer (50 mM Tris-HCl pH 8.0, 20% Guanidine Hydrochloride, 10 mM EDTA, 10 mM DTT, 0.4 μM PMSF) and, after addition of 0.05% Triton X-100, centrifuged at 12,000 x *g* for 10 min. The supernatant was used for protein determination by the Bradford assay (Bradford, 1976) following the manufacturer's instructions (Bio-Rad). Dilutions of bovine serum albumin were used to prepare a series of protein standards. Total protein was measured by Lowry analysis with a commercially available kit (Sigma-Aldrich).

Immunoblot analyses

The steady-state level of selected proteins in *Chlamydomonas* was examined by western blotting as previously described (van Dijk et al., 2005). Histone H3 and Ribosomal Protein S16 were detected with commercially available antibodies (ab1791 and ab26159, respectively; Abcam). The antibody against the large subunit of Rubisco was a generous gift of Robert Spreitzer (University of Nebraska-Lincoln) whereas the antibody against tryptophan synthase β-subunit was kindly provided by Thomas McKnight (Texas A&M University).

Semi-quantitative RT-PCR assays

Total RNA was isolated from *Chlamydomonas* cells with TRI reagent (Molecular Research Center), in accordance with the manufacturer's instructions, and treated with DNase I (Ambion) to remove contaminating DNA. Reverse transcription reactions were carried out as previously described (Carninci *et al.,* 1998) and the synthesized cDNAs were then used as template in standard PCR reactions (Sambrook and Russell, 2001). The numbers of cycles showing a linear relationship between input RNA and the final product were determined in preliminary experiments. Most primers were designed to match exonic sequences flanking one or more introns to distinguish contaminating PCR products possibly generated by amplification of any remaining DNA. Controls also included the use as template of reactions without reverse transcriptase and verification of PCR products by hybridization with specific probes (data not shown). The PCR conditions for amplification of most templates were 30 cycles at 94°C for 30 s, at 56°C for 30 s, and at 72°C for 20 s. Five-μl aliquots of each RT-PCR reaction were resolved on 1.5% agarose gels and visualized by ethidium bromide staining. The primer sequences were as follows: for *DGAT1* 511566-RT-F1, 5'-ACTGGTGGAATGCGGCTAC-3' and 511566-RT-R1 5'-TAGCAGCTCGTGGAACACAG-3'; for *DGTT1* 285889-RT-F1 5'-GAAGCAGGTGTTTGGCTTCT-3' and 285889-RT-R1 5'-CAGTGCCTCCGTGTAGGTCT-3'; for *DGTT2* 184281-RT-F1 5'-GCGCCGCAACATTTACATGG-3' and 184281-RT-R1 5'-CAGCCGTACTCGGTCTTGTG-3'; for *DGTT3* 188937-RT-F1 5'-GTCAGAGCCAAGTGCTGGAC-3' and 188937-RT-R1 5'-TCCACCTCCTTGTCGAACTC-3'; for *DGTT4* 190539-RT-F1 5'-

GCATGTTTGGGCAGTACGGC-3’ and 190539-RT-R1 5’-GCCTTGTGCTTGTCGTACAG-3’; for *DGTT5* 141301-RT-F1 5’-AGTCACTGCAGCAGCTGTCG-3’ and 141301-RT-R1 5’-GCCCACACACATCATGAGCG-3’; for *KASI* 205887-RT-F1 5’-CAGTGTGCTGCGGAATGC-3’ and 205887-RT-R1 5’-GGTCACACAAACACACATTTGA-3’; for *KAR* 335991-RT-F1 5’-GTCATCGGCCTGACCAAG-3’ and 335991-RT-R1 5’-ATGCCCTTGAGGATGGTCTC-3’; for *ACT1* ACT-Cod-F 5'-GACATCCGCAAGGACCTCTAC-3' and ACT-Cod-R 5'-GATCCACATTTGCTGGAAGGT-3'; for *COX4* 187882-RT-F1 5’-GGATAAGTTCGGCACTGAGG-3’ and 187882-RT-R1 5’-CGCCGACCTTCTTGATGTAC-3’ and for *COX12* 195712-RT-F1 5’-CACTGCTTTGTGCGATTCAA-3’ and 195712-RT-R1 5’-TTAGTACTTGCCGGTCCACA-3’.

Results

Cell growth and triacylglycerol accumulation in C. reinhardtii subject to nitrogen deprivation

To analyze the effect of nitrogen (N) depletion on *Chlamydomonas* grown under photoautotrophic conditions, we pre-cultured the wild type CC-125 strain to middle logarithmic phase in high salt (HS) minimal medium (Harris, 1989). Cells were then centrifuged, resuspended in either HS or HS-N medium at a density of 0.5 x 10^6 cells mL^{-1} and grown under photoautotrophic conditions for 6 days. Cultures were sampled daily

for specific analyses. In nitrogen replete medium cell density increased ~8-fold during the examined period whereas in medium lacking nitrogen cells approximately doubled in number within the first 48 h followed by an arrest in cell division (Fig. 3.1A). As reported for *Chlamydomonas* grown in medium containing acetate (Work et al., 2010), we also noticed that the average cell size increased during acclimation to nitrogen starvation (Fig. 3.1B) presumably as a consequence of the greater accumulation of carbon storage compounds (see below).

To assess lipid accumulation during the growth period, cells were examined by fluorescence microscopy after staining with the nonpolar lipid fluorophore Nile Red (Greenspan et al., 1985; Chen et al., 2009). Lipid body formation, as revealed by Nile Red fluorescence, increased significantly in nitrogen-stressed cells relative to those cultured in nutrient-replete medium (Fig. 3.1B). We also analyzed lipid-derived fatty acid methyl esters (FAMEs), by gas chromatography-flame ionization detection (GC-FID), to evaluate if the detected increase in nonpolar lipids during nitrogen deprivation corresponded to TAGs. Total cellular lipids were extracted and either derivatized and quantified by GC-FID or separated by thin layer chromatography (TLC). The TAG fraction, identified by co-migration with a purified TAG standard, was recovered from the TLC plate and its fatty acid content and composition also analyzed by GC-FID. Total fatty acid (FA) content per cell remained relatively constant, and similar to that of cells grown in nutrient replete medium, during the first two days of nitrogen starvation (Fig. 3.2A). However, FA levels showed an increase after 72 h in nutrient-depleted medium and doubled after 6 day of nitrogen deprivation. The proportion of FAs in TAGs also increased significantly in nitrogen-stressed cells (Fig. 3.2B), representing ~70% of the

total fatty acids in the cells by the end of the examined period (Fig. 3.2C).

The fatty acid composition of *C. reinhardtii* subject to nitrogen starvation for 6 days was similar to that of the predominant TAG fraction (Table 3.1 and Table 3.3). Cells grown in nutrient replete medium were rich in polyunsaturated FA species characteristic of membrane lipids, in particular 16:4Δ4,7,10,13 and 18:3Δ9,12,15 (α-linolenic acid) (Table 3.1). In contrast, nitrogen starved cells showed increased abundance of saturated fatty acids and of those with lower degree of unsaturation such as 16:0 (palmitic acid), 18:1Δ9 (oleic acid) and 18:2Δ9,12 (linoleic acid) (Table 3.1). This compositional bias, similar to that of many plant oils (Cahoon and Schmid, 2008), was even more pronounced in the TAG fraction of cells starved for nitrogen during 6 days (Table 3.1 and Table 3.3).

Changes in starch, chlorophyll and protein content in C. reinhardtii subject to nitrogen deprivation

To characterize further the metabolic changes triggered by nitrogen shortage under photoautotrophic conditions, we also analyzed starch, chlorophyll and soluble proteins in nutrient-stressed cells. Starch accumulation occurred very rapidly upon incubation in nitrogen depleted medium, increasing by ~14-fold after two days of nutrient deprivation and reaching maximal level one day latter, at ~19-fold the amount of starch measured in cells cultured in regular HS medium (Fig. 3.3A). Interestingly, the majority of starch synthesis appears to take place prior to the most significant accumulation of TAGs (cf., Figs. 3.3A and 3.2B).

Nitrogen-starved cells had a yellowish appearance compared to those grown under nutrient replete conditions, resulting from a marked decrease in chlorophyll content

with time of exposure to nitrogen depleted medium (Fig. 3.3B). Indeed, cells incubated in nitrogen free medium for 6 days had ~20% the chlorophyll amount measured in cells cultured in standard HS medium. Similarly, the content of total and soluble proteins also decreased substantially in nutrient deprived cells (Fig. 3.3C). *Chlamydomonas* cells subjected to nitrogen deprivation for 6 days had ~26% of the total protein amount in cells grown under nutrient replete conditions. Immunoblot assays also revealed a pronounced reduction, triggered by nitrogen shortage, in specific proteins such as the chloroplast located large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (involved in CO_2 fixation) and tryptophan synthase β-subunit (involved in tryptophan biosynthesis) as well as in subunits of the cytosolic ribosome, like ribosomal protein S16 (Fig. 3.4). These three polypeptides decreased to between 25 and 30% of their normal level after 6 days of nutrient depletion. In contrast, nitrogen starvation only caused a minor reduction in the steady-state amount of other proteins, such as histone H3 (Fig. 3.4). These observations, taken together, are consistent with progressive loss of certain plastidial functions, such as photosynthesis and amino acid biosynthesis, and of cytosolic protein translation capabilities triggered by nitrogen depletion. This interpretation is also in agreement with the reported changes in mRNA abundance in a recent transcriptome analysis of nitrogen-starved *Chlamydomonas* cells under photoheterotrophic conditions (Miller et al., 2010). However, histone proteins, that may be critical for preserving chromatin organization, appear to be much less affected by nutrient deprivation.

Triacylglycerol and starch accumulation in Coccomyxa sp. C-169 subject to nitrogen deprivation

To begin assessing the representativeness of *Chlamydomonas* as a model system

for studying metabolic processes triggered by nutrient starvation in green microalgae, we also sampled lipid and starch content in cells of *Coccomyxa* sp. C-169 experiencing nitrogen depletion. *Coccomyxa* belongs to the class Trebouxiophyceae (together with species of the genus *Chlorella*) within the green algae and it is quite divergent phylogenetically from *Chlamydomonas* which groups with members of the class Chlorophyceae (Palmqvist et al., 1997; Zoller and Lutzoni, 2003; Smith et al., 2011). In addition, strain C-169 originated from Marble Point Antarctica (Holm-Hasen, 1964) and is representative of algae adapted to an extreme environment as opposed to *Chlamydomonas* CC-125 which was reportedly collected from a potato field in Amherst, MA (Harris, 1989). Thus, we reasoned that if similar metabolic responses to nitrogen deprivation are observed in *Coccomyxa* and *Chlamydomonas* they might be indicative of processes conserved in a wide spectrum of green microalgae. However, *Coccomyxa* cells are smaller and grow slower, photoautotrophically under ambient levels of CO_2, than those of *Chlamydomonas* (data not shown). This limitation, which among other species-specific differences may be related to the lack of a carbon concentrating mechanism in *Coccomyxa* (Palmqvist et al., 1997), required longer incubation times in nitrogen free medium to assess metabolic changes triggered by nutrient depletion.

As observed in *Chlamydomonas*, *Coccomyxa* cells examined by fluorescence microscopy, after staining with the nonpolar lipid fluorophore Nile Red, also showed a significant increase in lipid body formation when subjected to nitrogen deprivation (Fig. 3.5A). The analysis of FAMEs by GC-FID revealed that total fatty acid content per cell remained relatively constant during the first four days of nitrogen starvation but did rise markedly after that, attaining an ~80% increase by the end of the examined period (Fig.

3.5B). The proportion of FAs in TAGs also augmented considerably in nitrogen-stressed cells (Fig. 3.5C), representing ~70% of the total fatty acids in the cells after 11 days of nutrient deprivation (Fig. 3.5D).

Coccomyxa cells grown in nutrient replete medium were rich in polyunsaturated FA species, in particular 18:2 and 18:3 (Table 3.2 and Table 3.4). In contrast, nitrogen starved cells showed a marked increase in the abundance of the monounsaturated FA 18:1 (Tables 3.2 and Table 3.4). These changes in FA composition were similar in trend to those occurring in *Chlamydomonas* under nitrogen deprivation that is an increase in fatty acids with lower degree of unsaturation (cf., Tables 3.1 and 3.2). However, there were also species-specific differences. In *Chlamydomonas* palmitic acid became the predominant FA in cells subject to nitrogen starvation whereas in *Coccomyxa* oleic acid showed the greatest abundance.

We also analyzed starch and chlorophyll content in nitrogen-stressed *Coccomyxa* cells. Starch synthesis occurred much rapidly than TAG accumulation, reaching a peak after 4 days of nitrogen deprivation (Fig. 3.6A). However, in relative terms, the amount of synthesized starch was much lower in *Coccomyxa* than in *Chlamydomonas* cells (cf., Figs. 3.3A and 3.6A). Nutrient shortage also resulted in a significant decrease in chlorophyll content in *Coccomyxa*. Cells incubated in nitrogen free medium for 11 days had ~25% of the chlorophyll amount measured in cells cultured in standard medium (Fig. 3.6B). One caveat with the *Coccomyxa* experiments is that the small cell size and solid cell wall made it difficult to break the cells and extract metabolites, reducing the accuracy of starch measurements and making the analysis of soluble proteins unreliable (data not shown). Nonetheless, our observations suggest that TAG and starch accumulation,

triggered by nitrogen depletion, followed similar overall patterns in *Chlamydomonas* and *Coccomyxa* grown photoautotrophically.

Expression of lipid biosynthesis genes in C. reinhardtii subject to nitrogen deprivation

Homologs of many proteins involved in eukaryotic lipid metabolic pathways are encoded in the *C. reinhardtii* genome (Riekhof et al., 2005; Merchant et al., 2007; Khozin-Goldberg and Cohen, 2011). However, their expression under nitrogen depletion is poorly understood, as is the case in most microalgae (Hu *et al.*, 2008; Miller et al., 2010; Khozin-Goldberg and Cohen, 2011). We examined steady-state transcript levels for a few lipid biosynthesis genes to assess whether their expression was consistent with the relatively late pattern of TAG accumulation under nitrogen stress (Fig. 3.2B). The *Chlamydomonas* genome contains six genes coding for diacylglycerol:acyl-CoA acyltransferases (DGATs), which catalyze the last step in the Kennedy pathway of TAG biosynthesis, the acylation of diacylglycerol to TAG (Weiss and Kennedy, 1956; Coleman and Lee, 2004; Courchesne et al., 2009). Based on primary sequence homology to functionally characterized eukaryotic enzymes, five genes encode DGAT2-like enzymes whereas the remaining one codes for a DGAT1 type isoform (Merchant et al., 2007; Khozin-Goldberg and Cohen, 2011). However, we were unable to detect transcripts, by reverse transcription-polymerase chain reaction (RT-PCR) assays, for one of the *DGAT2* genes (*DGTT5*; Prot ID: 536379) (data not shown). The remaining *DGAT2* like genes, *DGTT1* (Prot ID: 536226), *DGTT2* (Prot ID: 519435), *DGTT3* (Prot ID: 523869) and *DGTT4* (Prot ID: 190539), as well as the unique *DGAT1* gene (Prot ID: 536378) were tested for expression during the nitrogen deprivation period by semi-quantitative RT-PCR (Fig. 3.7).

While this manuscript was being prepared, Miller et al. (2010) reported a transcriptome study of *C. reinhardtii* subject to nitrogen deprivation under photoheterotrophic conditions. Their analysis focused predominantly on changes in transcript abundance occurring at 48 h after nitrogen depletion in medium containing acetate. Under these conditions, *DGTT1* showed a large increase in steady-state transcript levels whereas the other *DGAT* genes showed only minor or no change in expression (Miller et al., 2010). We also observed, under strictly photoautotrophic conditions, early and substantial up-regulation of *DGTT1* in cells subject to nitrogen starvation (Fig. 3.7). Additionally, *DGTT3* and *DGTT4* were also expressed at significantly higher levels, in particular after 6 days of nitrogen deprivation (Fig. 3.7). These observations suggest that *DGTT1* may contribute to TAG synthesis early during nitrogen starvation but several other *DGAT* genes may also play a role during the period of maximal TAG accumulation under photoautotrophic conditions. Intriguingly, DGAT1-like enzymes are primarily involved in TAG biosynthesis in seeds of *Arabidopsis thaliana* (Zhang et al., 2009) but the only gene encoding this type of isoform in *Chlamydomonas* was expressed at relatively low levels and not affected by nitrogen depletion (Fig. 3.7).

We also examined transcript abundance for two genes involved in *de novo* fatty acid synthesis. KASI, 3-Ketoacyl-ACP Synthase I, is a component of the multimeric fatty acid synthase II (FASII) and catalyzes the acyl-acyl carrier protein (acyl-ACP) dependent elongation steps from C4 to C16 in higher plants (Baud and Lepiniec, 2010; Miller et al., 2010). The only gene in the *Chlamydomonas* genome coding for KASI (*KASI*; Prot ID: 205887) was found to decrease in expression, under photoautotrophic conditions, in cells subject to nitrogen depletion (Fig. 3.7). KAR, 3-Ketoacyl-ACP Reductase, is also part of

the FASII complex and reduces the carbon 3 ketone to a hydroxyl group during FA synthesis (Baud and Lepiniec, 2010). This enzyme is also encoded by a single gene in the *Chlamydomonas* genome (*KAR*; Prot ID: 335991) and its transcript abundance decreased slightly in nitrogen deprived cells (Fig. 3.7). These results were somewhat surprising since doubling of the FA content in cells starved for nitrogen (Fig. 3.2A) is strongly suggestive of *de novo* fatty acid synthesis. Moreover, experiments with cerulenin, a specific inhibitor of the 3-ketoacyl-ACP synthase of FASII, have also implicated *de novo* FA synthesis in TAG accumulation in nitrogen-deprived *Chlamydomonas*, albeit under photoheterotrophic conditions (Fan et al., 2011). Interestingly, in the transcriptome analysis of Miller et al. (2010) the *KASI* mRNA increases by ~2-fold after 48 h of nitrogen deprivation in acetate containing medium. As discussed below, FASII enzymatic activity may not be limiting for TAG accumulation under our experimental conditions but differences in substrate availability under photoautotrophic or photoheterotrophic conditions may cause variations in the response of algal metabolic pathways to nitrogen depletion.

Discussion

Chlamydomonas reinhardtii accumulates both starch and TAGs when subject to a number of stresses such as nitrogen deprivation, high salinity, sulfur depletion or when exposed to high light (Klein, 1987; Ball et al., 1990; Matthew et al. 2009; Wang et al., 2009; Dean et al., 2010; Doebbe et al., 2010; Li et al., 2010; Moellering and Benning, 2010; Work et al., 2010; Fan et al., 2011; James et al., 2011; Siaut et al., 2011). Several

groups have begun characterizing the metabolic pathways involved in TAG synthesis in nutrient stressed cells of this organism (Wang et al 2009; Dean et al 2010; Li et al 2010; Miller et al 2010; Moellering and Benning 2010; Work et al 2010; Fan et al 2011; Siaut et al 2011). However, most studies have been performed under photoheterotrophic conditions (Wang et al 2009; Li et al 2010; Miller et al 2010; Moellering and Benning 2010; Work et al 2010; Fan et al 2011; James et al., 2011; Siaut et al 2011). In this case, acetate in the medium appears to contribute substantially to the accumulated TAGs (Fan et al 2011) and, possibly, to the synthesized starch (Ball et al., 1990; Work et al 2009). *Coccomyxa* species that participate, as lichen photobionts, in symbiotic associations with fungi are also capable of accumulating TAGs but the effect of environmental factors on lipid metabolism is poorly understood in these algae (Guschina et al., 2003).

Microalgae are considered a promising source of renewable biofuels. Yet, for the production of algal biofuels to become economically feasible, various analyses have underlined the necessity of low-cost culture systems, using sunlight as the sole or main energy source for biomass synthesis (Hu et al., 2008; Griffiths and Harrison, 2009; Rodolfi et al., 2009; Scott et al., 2010; Wijffels and Barbosa, 2010). Thus, we examined metabolite accumulation in *Chlamydomonas* and *Coccomyxa* cells subject to nitrogen deprivation under strictly photoautotrophic conditions, so that the information gained may be useful for understanding the biology of production strains (Morowvat et al., 2010). Nutrient depletion led to an arrest in cell division and an increase in starch and TAG content. In the absence of the nitrogen necessary for protein synthesis and cell growth, excess carbon from photosynthesis appears to be channeled into storage molecules, such as starch and TAGs. However, a detailed time course analysis of the

metabolic changes triggered by nitrogen depletion suggests that turnover of non-lipid cellular components may also play a role in TAG accumulation. The involvement of two processes, photosynthesis and recycling of previously assimilated carbon, in the synthesis of lipids in response to silicon deficiency has also been proposed for the diatom *Cyclotella cryptica* (Roessler, 1988).

Upon nitrogen deprivation *Chlamydomonas* cells initially accumulate starch, which increases its content ~14-fold during the first two days of stress (Fig. 3.3A). Starch biosynthesis likely involves newly fixed carbon through photosynthesis since chlorophyll and soluble protein contents remain relatively high at the beginning of the nutrient deprivation (Figs. 3.3B and 3.3C). In contrast, there is virtually no change in total fatty acid levels on a per cell basis during this period (Fig. 3.2A). Nonetheless, since the cells almost double in number in the first 48 h of stress (Fig. 3.1A), maintaining a steady-state amount of fatty acids per cell implies the occurrence of *de novo* synthesis. The majority of the FAs are likely devoted to preserving membrane homeostasis but a small fraction may also be used for incipient TAG biosynthesis (Fig. 3.2B). Although our data cannot discriminate whether FAs employed in TAG production are synthesized *de novo* and/or recycled from pre-existing lipids such as those of the plastid membranes (Wang et al., 2009; Siaut et al., 2011).

At latter time points, *Chlamydomonas* cells show clear accumulation of total fatty acids (Fig. 3.2A) and a significant increase in TAG content (Figs. 3.2B and 3.2C). Conversely, starch levels display a slight decline after prolonged incubation in nitrogen deprived medium, which becomes more obvious after ten days of nutrient stress (data not shown). These observations suggest considerable *de novo* fatty acid synthesis in cells

depleted of nitrogen for several days, but the synthesized lipids are unlikely to derive from newly (photosynthetically) assimilated carbon since the chlorophyll and soluble protein contents per cell (including enzymes like Rubisco that are essential for CO_2 fixation) are greatly diminished at these time points (Figs. 3.3B, 3.3C and 3.4). Indeed, a decrease in photosynthesis and overall anabolic processes has been reported in nitrogen starved *Chlamydomonas* cells under both photoautotrophic and photoheterotrophic conditions (Martin and Goodenough, 1975; Bulte and Wollman, 1992; Li et al., 2010; Miller et al., 2010; Work et al., 2010). Thus, in *C. reinhardtii* cultured under photoautotrophic conditions in nitrogen depleted medium, fatty acids for TAG biosynthesis may be partly obtained at the expense of the carbon assimilated in other cellular components, to some degree starch and chlorophyll but also proteins (Figs. 3.3C and 3-4) and ribosomal RNA since there is clear evidence for ribosome degradation (Martin and Goodenough, 1975; Martin et al., 1976; data not shown). Similar temporal changes in starch, TAG and chlorophyll contents triggered by nitrogen deprivation were observed in *Coccomyxa* sp. C-169, which is phylogenetically quite divergent from *Chlamydomonas*, suggesting that these metabolic patterns may be shared by at least a subset of green microalgae. Moreover, conversion of previously assimilated carbon in the form of starch to neutral lipids, under nitrogen limited conditions, has also been proposed for the oleaginous green alga *Pseudochlorococcum* sp. (Li et al., 2011).

Coccomyxa cells cultured in nitrogen deprived medium accumulated less starch, in relative terms, than *Chlamydomonas* but a similar proportion of TAGs, representing ~70% of the total fatty acids in the cells after 11 days of nutrient deprivation (Figs. 3.5 and 3.6). The predominant fatty acid in *Coccomyxa* triacylglycerols was oleic acid

whereas palmitic acid showed the greatest abundance in *Chlamydomonas*. Interestingly, despite these species-specific differences, both *Coccomyxa* sp. C-169 and *C. reinhardtii* CC-125 displayed similar trends in the accumulation of starch and TAGs and in the reduction of chlorophyll content triggered by nitrogen deprivation. Given the substantial divergence in habitat and phylogeny between these algal species, our observations suggest that certain metabolic responses to N shortage may be shared by a broad range of green microalgae.

TAG biosynthesis can occur by several enzymatic mechanisms in eukaryotes (Hu et al., 2008; Baud and Lepiniec, 2010; Khozin-Goldberg and Cohen, 2011). In the Kennedy pathway, DGATs catalyze the acylation of diacylglycerol to TAGs (Weiss and Kennedy, 1956; Coleman and Lee, 2004; Courchesne et al., 2009). Although the specific mechanisms of TAG synthesis in microalgae are poorly characterized, increased abundance of the transcripts for several DGAT homologs (this work and Miller et al., 2010), particularly at latter time points during nitrogen starvation, suggests that these enzymes may play a role in *Chlamydomonas*. In contrast, transcripts for KASI and KAR, subunits of the fatty acid synthase II complex (Baud and Lepiniec, 2010), appear to decrease as cells are incubated photoautotrophically in nitrogen depleted medium. These results are counterintuitive since doubling of the FA content in nitrogen starved *Chlamydomonas* cells is strongly suggestive of *de novo* fatty acid synthesis (Fig. 3.2A) and experiments with cerulenin, an inhibitor of KASI, have implicated *de novo* FA synthesis in TAG accumulation in medium containing acetate (Fan et al., 2011). Additionally, *KASI* appears to be up-regulated when cells are subject to nitrogen depletion in the presence of acetate (Miller et al., 2010). With the caveat that mRNA

levels do not necessarily reflect protein amounts or enzymatic activities, we hypothesize that expression of (some) genes encoding FASII enzymes may be modulated by substrate availability. In this context, normal FASII activity may not be limiting for TAG accumulation under photoautotrophic conditions, as precursors for fatty acid synthesis may be scarce, likely derived at least in part from the recycling of previously assimilated carbon in proteins, ribosomal RNAs, chlorophyll and possibly starch. However, *KASI* up-regulation may be necessary when exogenous acetate provides an abundant precursor for lipid synthesis (Fan et al., 2011). This interpretation is consistent with the ~35% greater accumulation of TAGs in cells subject to nitrogen deprivation for 6 days in acetate containing medium in comparison with those cultured in minimal medium (data not shown).

The primary product of CO_2 fixation, 3-phosphoglycerate, feeds directly into the starch biosynthesis pathway but it can also be used as a precursor of acetyl-CoA for fatty acid synthesis and of the glycerol backbone of TAGs (Hu et al., 2008; Radakovits et al., 2010). This prompted several groups to investigate whether biosynthesis of starch and TAGs compete with each other and whether *Chlamydomonas* mutants defective in starch synthesis would accumulate higher levels of lipids under nitrogen starvation (Wang et al., 2009; Li et al., 2010; Work et al., 2010; Siaut et al., 2011). Despite some promising results (Wang et al., 2009; Li et al., 2010; Work et al., 2010) the relationship between these two metabolic pathways appears to be more complex than mere competition (Work et al., 2010; Li et al., 2011; Siaut et al., 2011), emphasizing our incomplete understanding of the interdependence and regulation of metabolic processes in microalgae. Additionally, our results suggest that recycling of other cellular components may also contribute

substantial carbon skeletons for TAG synthesis under nitrogen depletion.

Nutrient deprivation is one of the common stresses encountered by microorganisms in nature (Elser et al., 2007). Under nutrient replete conditions, growth is promoted via increased transcription and translation, processes that require large amounts of ribosomes and other anabolic structural components (Warner, 1999). Indeed, proteins are the dominant fraction in the biomass of fast-growing photosynthetic organisms (Langner et al., 2009; Huo et al., 2011). In contrast, under nutrient depletion, growth is inhibited and most of the anabolic machinery becomes superfluous (Acquisti et al., 2009). In this context, anabolic structural components, such as ribosomes and chlorophyll, may be targeted for degradation and nutrient recycling (Kraft et al., 2008). When exogenous nitrogen is depleted, selective autophagic processes may function to make available endogenous nitrogen for limited *de novo* protein synthesis so that cells can adapt and change their fate, including in the case of *Chlamydomonas* differentiation into sexual gametes and acquisition of the ability to survive a prolonged nutrient stress (Martin and Goodenough, 1975; Ball et al., 1990; Wang et al., 2009). Recycled excess carbon appears to accumulate in nitrogen-poor storage compounds such as TAGs. A greater understanding of these processes may provide targets for the metabolic engineering of algal strains with increased capacity to convert their biomass into useful biofuel precursors.

Acknowledgements

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Figures

Figure 3.1 Growth and neutral lipid accumulation of *Chlamydomonas reinhardtii* CC-125 subject to nitrogen deprivation. Cells were cultured photoautotrophically, for the indicated times, in High Salt medium (HS+N) or in the same medium lacking nitrogen (HS-N). (A) Growth curves displaying changes in cell density over time. Each data point represents the average of three independent experiments ± s.e. (B) Fluorescence microscopy detection of neutral lipid accumulation by Nile Red staining. The images shown are representative of typical cells at the different time points. In each medium series, the upper panels correspond to merged transmitted light and fluorescent images whereas the lower panels correspond to fluorescent images. Scale bars equal 10 μm.

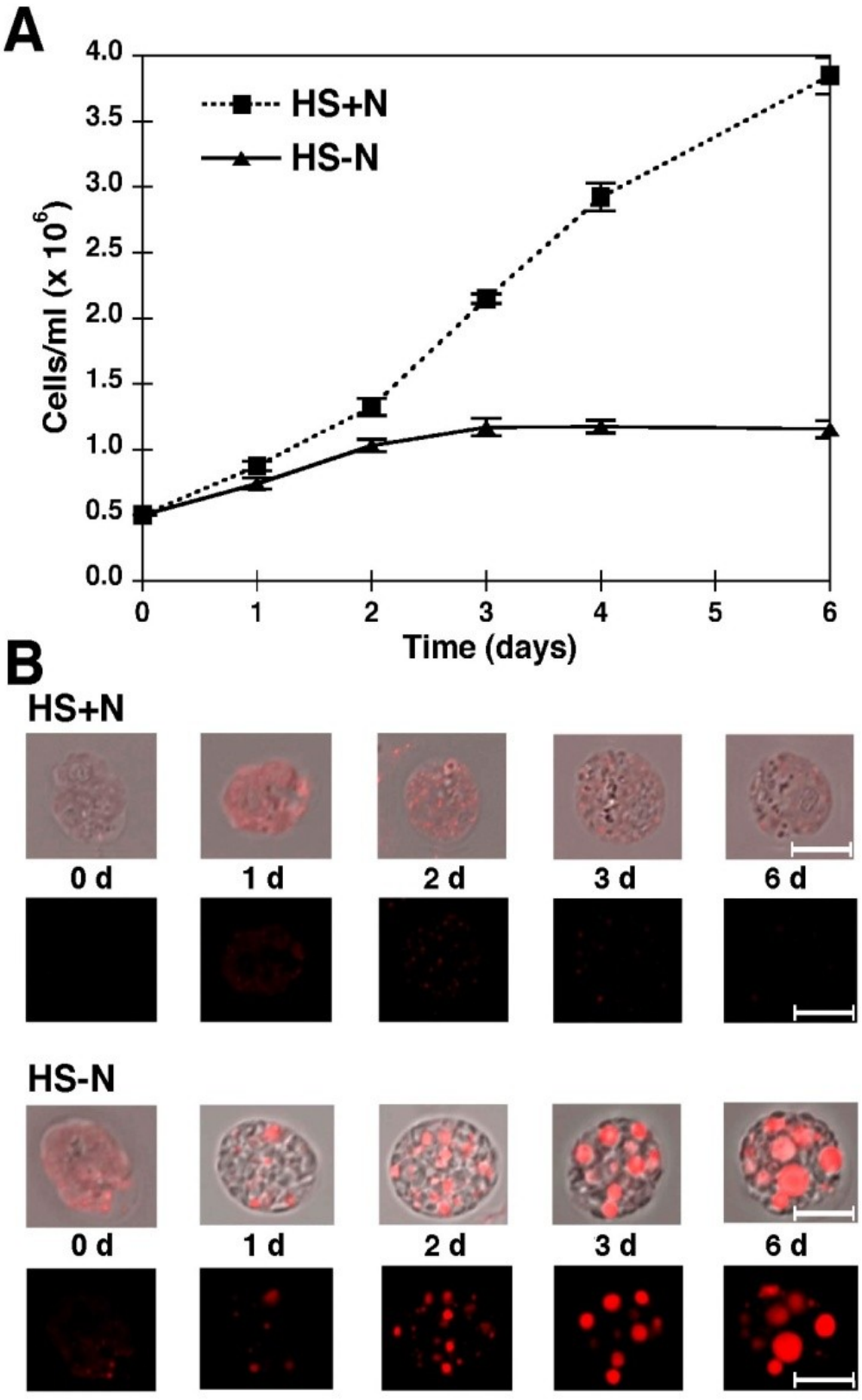

A
HS+N
HS-N
Cells/ml (x 10^6)
Time (days)
B
HS+N
0 d
1 d
2 d
3 d
6 d
HS-N
0 d
1 d
2 d
3 d
6 d

Figure 3.2 Total fatty acid and TAG accumulation in *Chlamydomonas* CC-125 subject to nitrogen deprivation under photoautotrophic conditions. Cells were incubated for the indicated times in HS medium, either nutrient replete (+N) or nitrogen depleted (-N). Values indicate the mean of three independent experiments ± s.e. (A) Total fatty acid content expressed as nanograms per 1,000 cells. (B) Fatty acids in TAGs expressed as nanograms per 1,000 cells. (C) Fatty acids in the TAG fraction expressed as percentage of the total FAs in a cell.

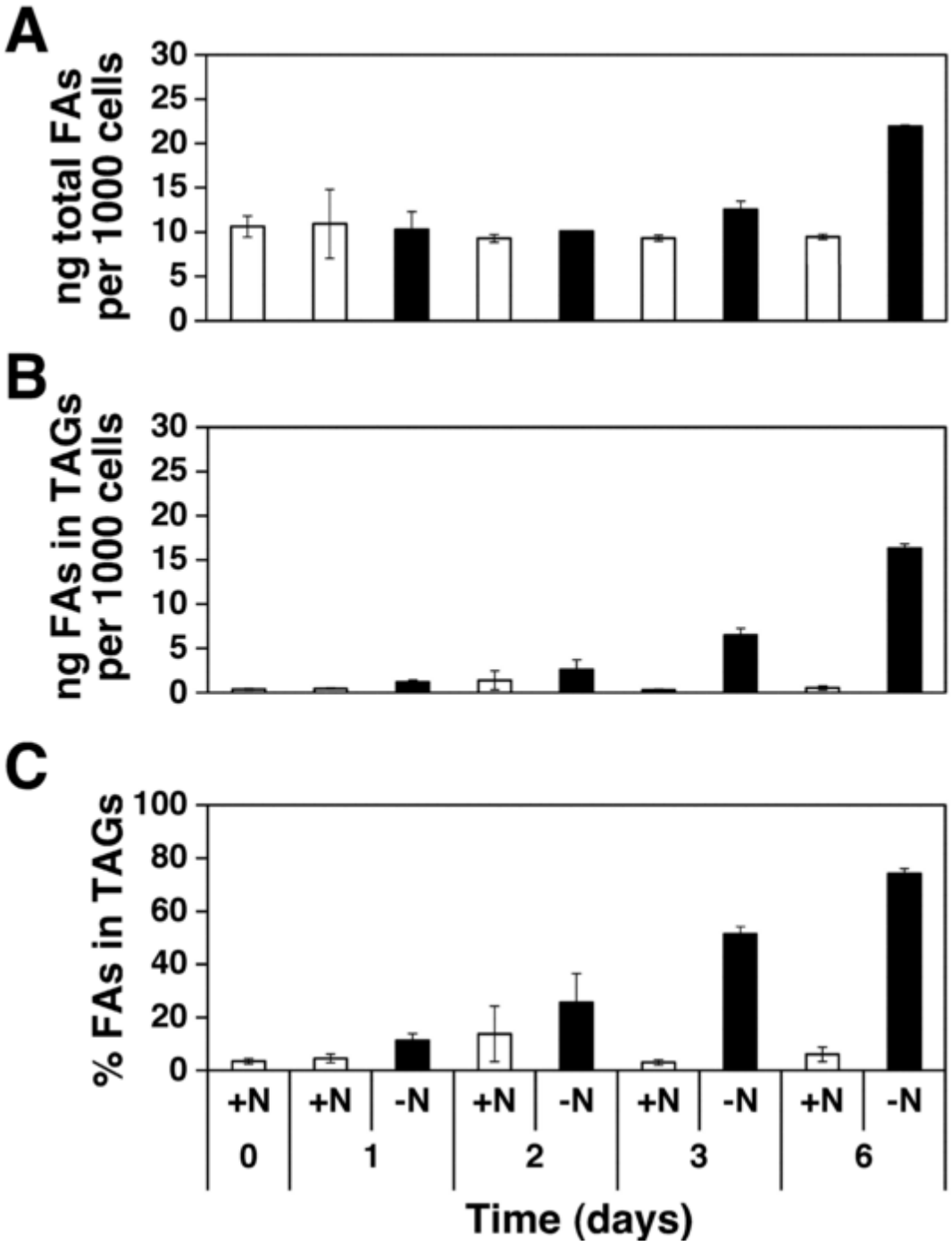

A
ng total FAs per 1000 cells
30
25
20
15
10
5
0
B
ng FAs in TAGs per 1000 cells
30
25
20
15
10
5
0
C
% FAs in TAGs
100
80
60
40
20
0
+N
+N
-N
+N
-N
+N
-N
+N
-N
0
1
2
3
6
Time (days)

Figure 3.3 Starch, chlorophyll and protein contents in *Chlamydomonas* CC-125 subject to nitrogen deprivation under photoautotrophic conditions. Cells were cultured for the indicated times in HS medium, either nutrient replete (+N) or nitrogen depleted (-N). Values indicate the mean of three independent experiments ± s.e. (A) Starch content expressed as nanograms per 1,000 cells. (B) Chlorophyll amount expressed as nanograms per 1,000 cells. (C) Total protein content expressed as nanograms per 1,000 cells. The hatched bars indicate soluble protein amounts.

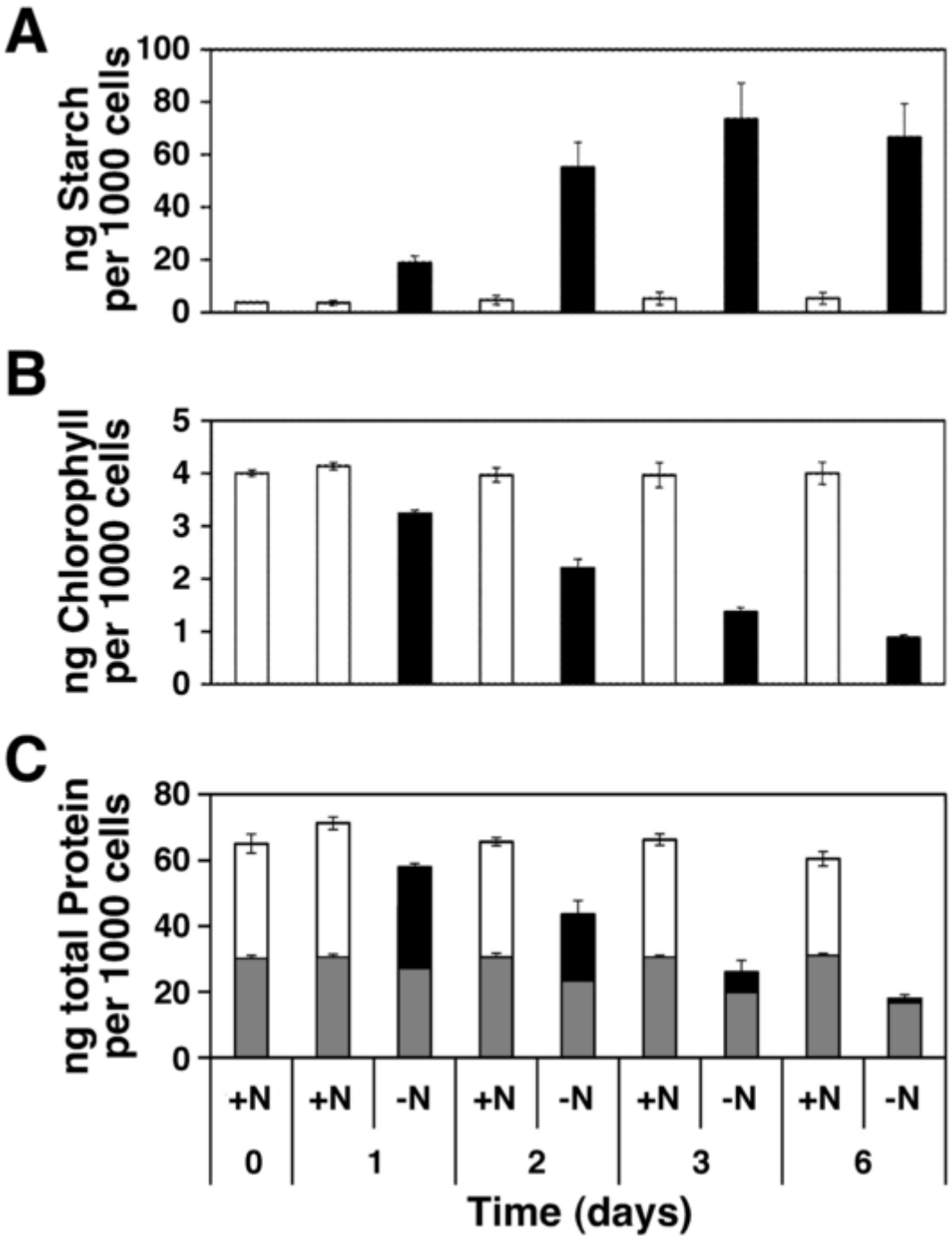

A
ng Starch per 1000 cells
100
80
60
40
20
0
B
ng Chlorophyll per 1000 cells
5
4
3
2
1
0
C
ng total Protein per 1000 cells
80
60
40
20
0
+N
+N
-N
+N
-N
+N
-N
+N
-N
0
1
2
3
6
Time (days)

Figure 3.4 Immunoblot analysis of specific polypeptides in *C. reinhardtii* CC-125 subject to nitrogen deprivation under photoautotrophic conditions. Cells were grown for the indicated times in HS medium with (+) or without (-) nitrogen. Whole cell protein extracts were separated by SDS-PAGE, transferred to nitrocellulose and probed with antibodies raised against Ribosomal Protein S16 (RPS16), Tryptophan Synthase β-subunit (TSβ), the large subunit of Ribulose-1,5-bisphosphate carboxylase/oxygenase (Lg Sub Rubisco) or histone H3 (H3). The panels show representative images from one out of three independent experiments. Numbers below the blots indicate the relative abundance of the examined proteins.

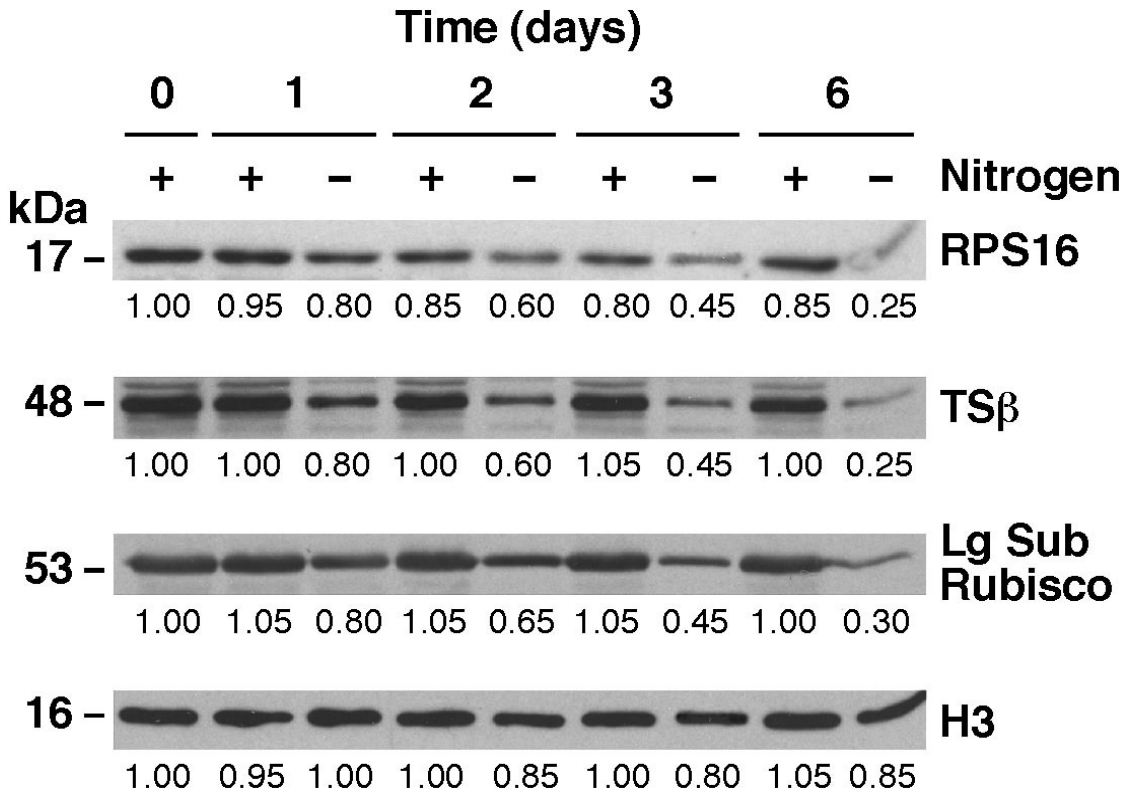


Figure 3.5 Lipid accumulation in *Coccomyxa* sp. C-169 subject to nitrogen deprivation under photoautotrophic conditions. Cells were incubated for the indicated times in BBM medium, either nutrient replete (+N) or nitrogen depleted (-N). (A) Fluorescence microscopy detection of neutral lipid accumulation by Nile Red staining. The images shown are representative of typical cells at the different time points. In each medium series, the upper panels correspond to merged transmitted light and fluorescent images whereas the lower panels correspond to fluorescent images. Scale bars equal 2.5 μm. (B) Total fatty acid content expressed as nanograms per 1,000 cells. (C) Fatty acids in TAGs expressed as nanograms per 1,000 cells. (D) Fatty acids in the TAG fraction expressed as percentage of the total FAs in a cell. Values indicate the mean of three independent experiments ± s.e.

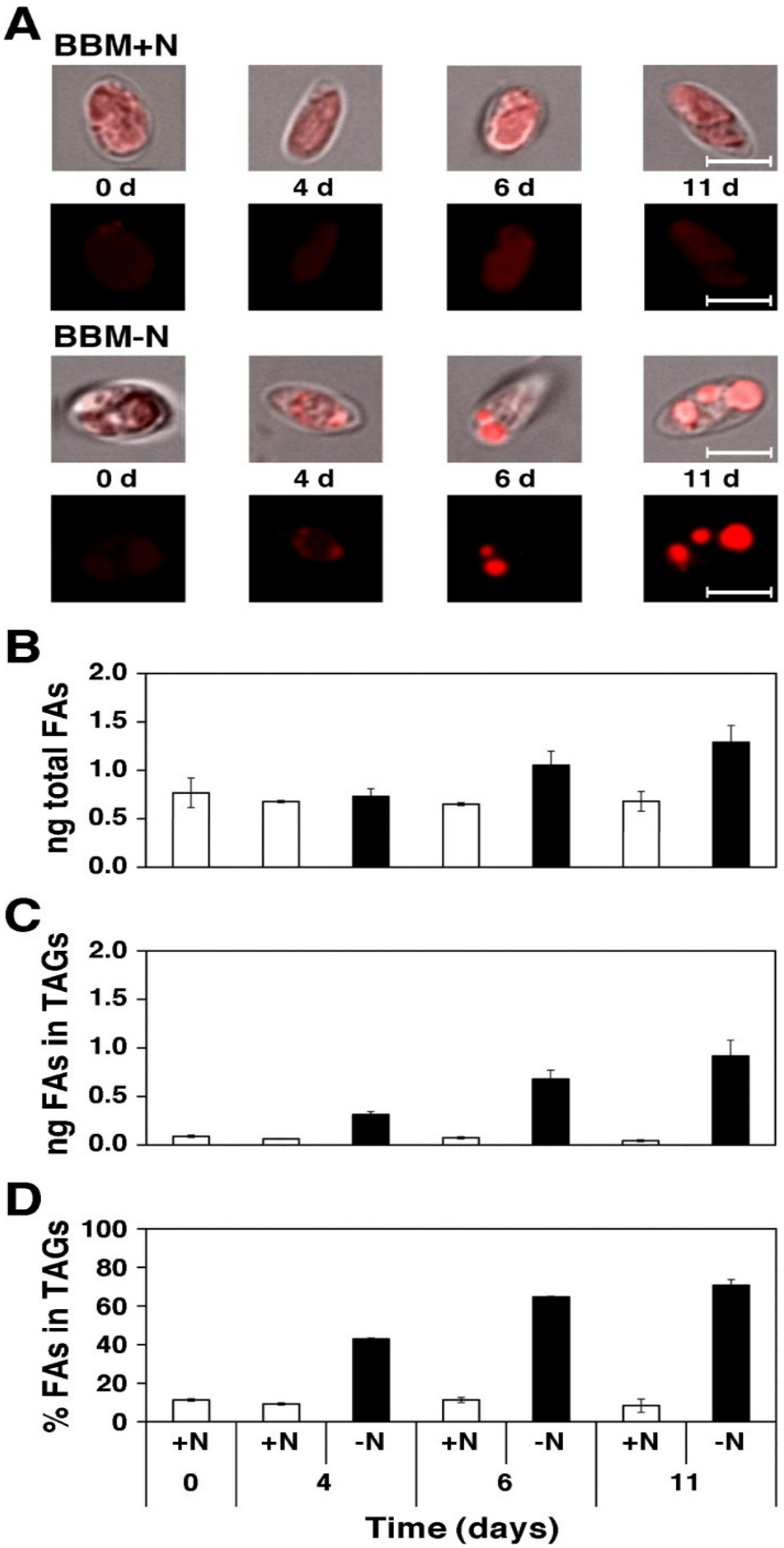

A
BBM+N
0 d
4 d
6 d
11 d
BBM-N
0 d
4 d
6 d
11 d
B
ng total FAs
2.0
1.5
1.0
0.5
0.0
C
ng FAs in TAGs
2.0
1.5
1.0
0.5
0.0
D
% FAs in TAGs
100
80
60
40
20
0
+N
+N
-N
+N
-N
+N
-N
0
4
6
11
Time (days)

Figure 3.6 Starch and chlorophyll contents in *Coccomyxa* sp. C-169 subject to nitrogen deprivation under photoautotrophic conditions. Cells were grown for the indicated times in BBM medium, either nutrient replete (+N) or nitrogen depleted (-N). (A) Starch content expressed as nanograms per 1,000 cells. Values correspond to the average of two independent experiments. (B) Chlorophyll amount expressed as nanograms per 1,000 cells. Values indicate the mean of three independent experiments ± s.e.

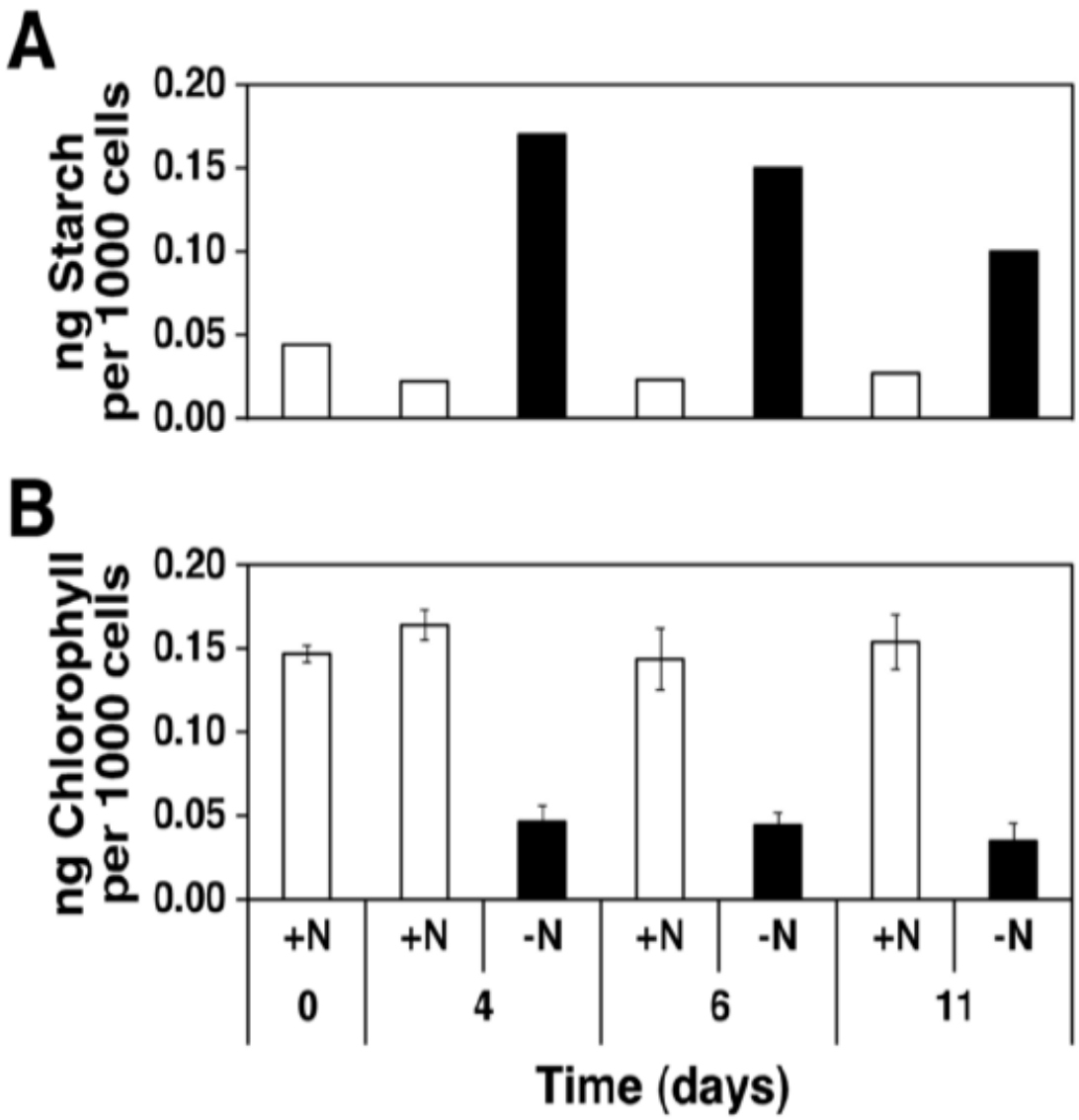
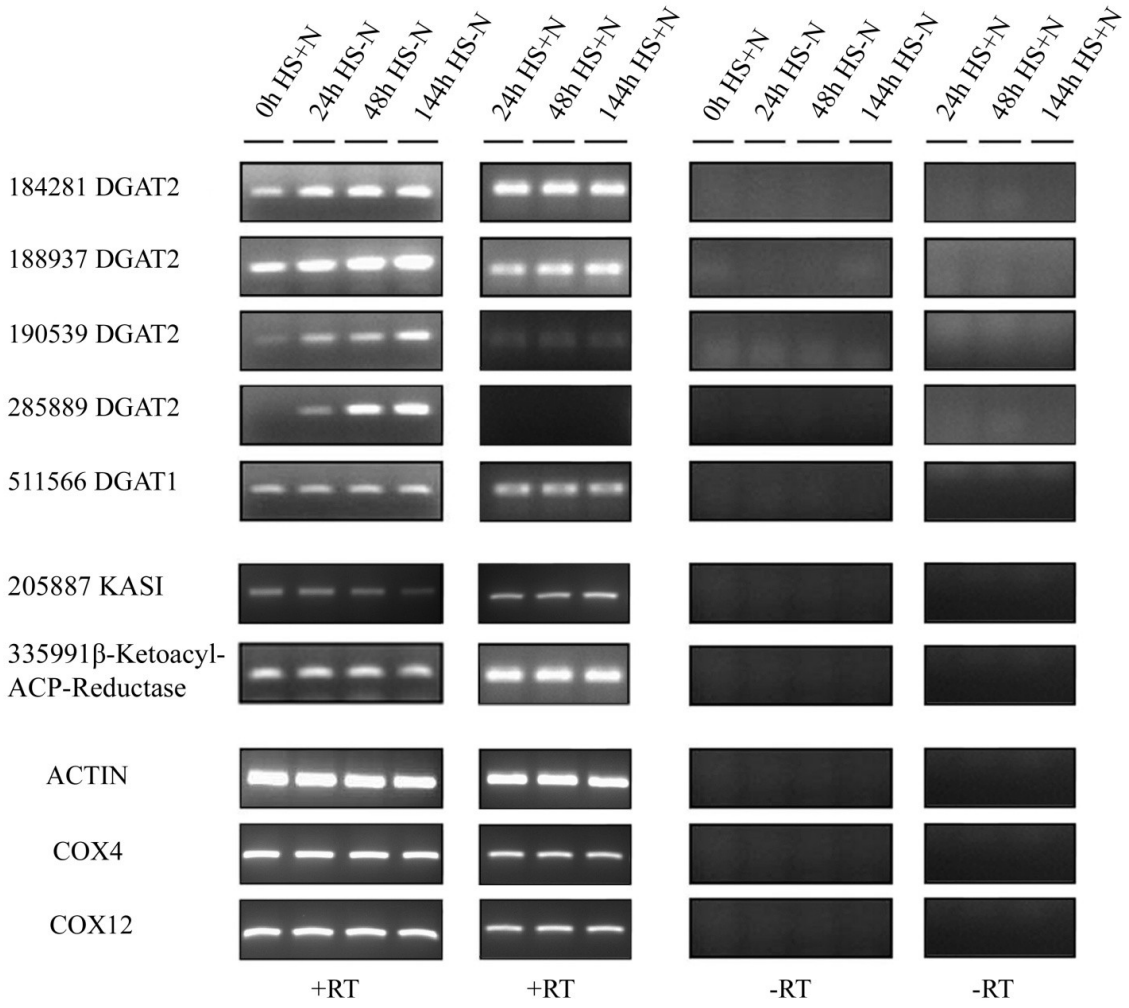


Figure 3.7 Expression of lipid biosynthesis genes in *Chlamydomonas reinhardtii* CC-125 subject to nitrogen deprivation under photoautotrophic conditions. Cells were grown for the indicated times in HS medium with (+N) or without (-N) nitrogen. Transcript abundance corresponding to specific genes involved in lipid synthesis was analyzed by semi-quantitative RT-PCR. Reactions were performed as described under experimental procedures in the presence (+RT) or absence (-RT) of reverse transcriptase. The panels show representative images of agarose resolved RT-PCR products stained with ethidium bromide. Examined genes included those encoding diacylglycerol:acyl-CoA acyltransferases type 2 (*DGTT1*, *DGTT2*, *DGTT3*, and *DGTT4*) and type 1 (*DGAT1*), 3-ketoacyl-ACP synthase I (*KASI*) and 3-ketoacyl-ACP reductase (*KAR*). Amplification of the mRNAs corresponding to *ACT1* (encoding actin), *COX4* (encoding mitochondrial cytochrome c oxidase subunit Vb) and *COX12* (encoding mitochondrial cytochrome c oxidase subunit VIb) were used as controls for equal amounts of input RNA and for the efficiency of the RT-PCR reactions.


0h HS+N
24h HS-N
48h HS-N
144h HS-N
24h HS+N
48h HS+N
144h HS+N
0h HS+N
24h HS-N
48h HS-N
144h HS-N
24h HS+N
48h HS+N
144h HS+N
184281 DGAT2
188937 DGAT2
190539 DGAT2
285889 DGAT2
511566 DGAT1
205887 KASI
335991β-Ketoacyl-ACP-Reductase
ACTIN
COX4
COX12
+RT
+RT
-RT
-RT

Tables

Table 3.1 Fatty acid composition (wt% of the total FAs ± SD, n=3) of the total lipid extract or purified triacylglycerols (TAGs) from *C. reinhardtii* cells cultured in HS+N or HS-N media for the indicated times.

Sample Name	16:0	16:1	16:3	16:4	18:0	18:1 Δ9	18:1 Δ11	18:2	18:3 Δ5,9,12	18:3	18:4 Δ5,9,12,15	Other
0d HS+N	17.0±0.2	3.4±0.2	3.1±0.1	20.2±0.3	2.2±0.1	2.7±0.1	3.8±0.2	5.3±0.7	5.5±0.2	31.9±0.4	3.7±0.3	1.1
1d HS-N	20.4±1.3	2.9±0.4	2.9±0.1	17.3±0.5	2.1±0.1	6.5±0.9	3.7±0.2	6.6±0.5	5.8±0.7	27.3±0.8	3.3±0.4	1.2
2d HS-N	21.9±3.4	2.7±0.5	3.1±0.2	14.8±3.3	2.3±0.2	8.1±2.2	3.9±0.4	7.9±1.1	5.9±0.1	24.9±3.0	3.0±0.3	1.5
3d HS-N	27.5±0.3	1.8±0.2	2.9±0.1	9.7±0.8	2.6±0.1	10.6±1.3	4.5±0.3	9.7±0.4	6.8±0.4	19.9±1.1	2.4±0.2	1.5
6d HS-N	30.6±0.6	1.7±0.1	2.7±0.1	6.9±0.2	2.9±0.1	12.1±0.5	4.4±0.3	12.3±0.2	6.6±0.2	16.1±0.6	2.1±0.2	1.6
6d HS-N (TAGs)	31.6±0.5	1.9±0.1	2.5±0.1	5.6±0.2	2.7±0.1	15.6±0.6	4.3±0.3	13.3±0.2	6.0±0.2	12.9±0.5	1.9±0.2	1.8

Table 3.2 Fatty acid composition (wt% of the total FAs ± SD, n=3) of the total lipid extract or purified triacylglycerols (TAGs) from *Coccomyxa* cells cultured in BBM+N or BBM-N media for the indicated times.

Sample Name	16:0	16:1	16:2	16:3	18:1	18:2	18:3	Other
0d BBM+N	17.5±0.7	8.7±1.0	7.8±0.5	10.8±0.8	7.5±0.1	20.8±0.3	23.6±2.4	3.3
4d BBM-N	18.1±0.7	1.1±0.5	2.9±0.1	5.6±0.1	30.4±0.2	20.2±0.3	17.4±0.5	4.3
6d BBM-N	17.0±0.1	0.8±0.1	1.7±0.3	4.6±0.3	40.1±2.6	16.6±1.1	14.8±0.9	4.5
11d BBM-N	16.1±0.4	1.3±0.2	1.5±0.0	3.9±0.1	44.3±1.7	15.1±0.2	13.1±0.1	4.7
11d BBM-N (TAGs)	11.4±0.3	0.4±0.0	0.7±0.1	2.5±0.1	57.7±0.3	13.7±0.2	10.3±0.3	3.2

Table 3.3 Content of fatty acids (FAs) in whole cells or in purified TAGs from *C. reinhardtii* cells maintained in HS+N or HS-N media for the indicated times. Values shown are expressed as ng FA/1000 cells ± SD (n=3).

Sample Name	16:0	16:1	16:3	16:4	18:0	18:1 Δ9	18:1 Δ11	18:2	18:3 Δ5,9,12	18:3	18:4 Δ5,9,12,15	Other
0d HS+N	1.82±0.22	0.36±0.02	0.33±0.04	2.14±0.22	0.23±0.02	0.29±0.03	0.40±0.02	0.58±0.14	0.59±0.09	3.38±0.34	0.39±0.01	0.13
1d HS-N	2.35±0.56	0.34±0.08	0.33±0.07	1.97±0.40	0.23±0.03	0.72±0.14	0.41±0.05	0.74±0.12	0.64±0.10	3.04±0.45	0.36±0.04	0.133
2d HS-N	2.20±0.35	0.28±0.05	0.32±0.02	1.49±0.33	0.23±0.02	0.82±0.22	0.39±0.04	0.79±0.11	0.60±0.01	2.51±0.29	0.30±0.03	0.146
3d HS-N	3.46±0.31	0.23±0.04	0.36±0.05	1.20±0.09	0.32±0.02	1.35±0.27	0.57±0.07	1.22±0.13	0.85±0.04	2.48±0.1	0.30±0.01	0.199
6d HS-N	6.71±0.18	0.38±0.02	0.59±0.01	1.51±0.05	0.64±0.01	2.65±0.12	0.96±0.05	2.71±0.06	1.45±0.04	3.53±0.13	0.45±0.04	0.351
6d HS-N (TAGs)	5.16±0.23	0.30±0.01	0.41±0.02	0.92±0.03	0.44±0.01	2.54±0.13	0.70±0.02	2.18±0.10	0.98±0.04	2.09±0.02	0.31±0.02	0.29

Table 3.4 Content of fatty acids (FAs) in whole cells or in purified TAGs from *Coccomyxa* cells maintained in BBM+N or BBM-N media for the indicated times. Values shown are expressed as ng FA/1000 cells ± SD (n=3).

Sample Name	16:0	16:1	16:2	16:3	18:1	18:2	18:3	Other
0d BBM+N	0.14±0.03	0.07±0.01	0.06±0.01	0.08±0.01	0.06±0.01	0.16±0.03	0.19±0.06	0.03
4d BBM-N	0.13±0.01	0.01±0.01	0.02±0.00	0.04±0.01	0.22±0.03	0.15±0.02	0.13±0.02	0.03
6d BBM-N	0.18±0.03	0.01±0.01	0.02±0.00	0.05±0.01	0.42±0.09	0.17±0.01	0.15±0.01	0.05
11d BBM-N	0.21±0.02	0.02±0.00	0.02±0.00	0.05±0.01	0.57±0.10	0.19±0.02	0.17±0.02	0.06
11d BBM-N (TAGs)	0.10±0.02	0.01±0.00	0.01±0.00	0.023±0.00	0.53±0.09	0.13±0.02	0.10±0.01	0.029

CHAPTER 4 - EFFECTS OF AUTOPHAGY-INDUCING AND AUTOPHAGY-INHIBITING COMPOUNDS ON THE ACCUMULATION OF TRIACYLGLYCEROL IN THE GREEN ALGAE *Chlamydomonas reinhardtii*

Abstract

Autophagy can serve as a protective mechanism against nutrient deprivation by recycling macromolecules and removing damaged organelles. 3-methyladenine (3-MA), a potent autophagy-inhibitor in animals and plants, induces triacylglycerol (TAG) accumulation in *Chlamydomonas reinhardtii* cells growing photoautotrophically in both nutrient replete (HS+N) and nitrogen-deprived (HS-N) media. 3-MA inhibits both formation of autophagosomes and cell death. On the other hand, treatment with rapamycin, an autophagy-inducer, does not significantly increase TAG accumulation in *Chlamydomonas* cells growing in both nutrient replete or nitrogen-deprived media. Thin layer chromatography (TLC) plate assay, a powerful tool in the analysis of lipids, shows stronger TAG bands with higher intensity when total lipid extract is used from *Chlamydomonas* cells growing for 6 days in nitrogen-deprived media and supplied with 1 mM 3-MA compared to control cells. Interestingly, 3-MA also induces TAG accumulation in *Chlamydomonas* cells growing in nutrient replete media. Moreover, data obtained by gas chromatography analysis determines a higher TAG content in cells supplied with 3-MA and growing in both control and N-deprived media. We conclude that TAG accumulation in nitrogen-deprived *Chlamydomonas reinhardtii* cells may not be due to induction of autophagic pathways by nutrient depetion.

Keywords: Autophagy, 3-methyadenine, triacylglycerol, rapamycin.

Introduction

Macroautophagy, often termed simply autophagy, is a nonselective catabolic membrane-trafficking process by which eukaryotic cells transfer organelles, organelle fragments, and cytosolic macromolecules into the vacuole/lysosome for degradation by proteases, lipases, nucleases, and acid hydrolases present inside this compartment (Bassham, 2009). It is also involved in other cellular events including differentiation and defense against parasites (Pérez-Pérez et al., 2010; Duszenko et al., 2011). During autophagy, entire organelles and protein complexes are enclosed in bulk within cup-shaped membranes that expand (Muntz, 2007) and fuse to completely surround the material, generating a double membrane vesicle known as the autophagosome (Carter et al., 2004). The autophagosomes appear to form *de novo* from small membrane structures generated from the endoplasmic reticulum or Golgi (Dunn et al., 2007) called the preautophagosomal structure (PAS) (Pérez-Pérez et al., 2010). Once the autophagosome is formed, its outer membrane fuses with the vacuole/lysosome membrane, forming the autolysosome (Pérez-Pérez et al., 2010). The material is then released into the lysosome where digestion by hydrolytic enzymes occurs (Cuervo 2004; Gozuacik and Kimchi 2004; Pérez-Pérez et al., 2010). All steps including the final lysosomal degradation of cytoplasmic materials are ATP-dependent (Blommaart et al., 1997; Kim and Klionsky, 2000; Klionsky and Emr, 2000; Bursch, 2001). Nutrients generated from the breakdown materials in the vacuole are presumably exported to the cytoplasm for re-use (Moriyasu and Ohsumi, 1996; Xie and Klionsky, 2007; Nakatogawa et al., 2009).

A basal level of autophagy occurs constitutively for the breakdown of unwanted cellular materials (Aubert et al., 1996; Inoue et al. 2006); it is dramatically enhanced in

response to stimuli from within the cell or from the environment (Duszenko et al., 2011). Such stimuli include nutrient deprivation, hypoxia (Cuervo, 2004), physiological signals such as hormones and growth factors (Méresse et al., 1999). The autophagic pathway appears to be conserved throughout eukaryotes (Mizushima et al., 1998; Doelling et al., 2002; Hanaoka et al., 2002; Bassham et al., 2006); it is mediated by AuTophaGy-related (*ATG*) genes identified and characterized in the yeast *Saccharomyces cerevisiae* (Klionsky et al., 2003). *ATG* encode more than 30 proteins that participate in autophagy regulation and autophagosome formation (Bassham et al., 2006; Bassham, 2007; Meijer et al., 2007; Pérez-Pérez et al., 2010). Orthologues have been identified in mammals, plants, fungi (Meijer et al., 2007; Duszenko et al., 2011), and more recently in the green algae *Chlamydomonas reinhardtii* (Pérez-Pérez et al., 2010). In the process of autophagy, major components that are conserved through evolution include two ubiquitin-like proteins ATG8 and ATG12, the three enzymes ATG3, ATG7, and ATG10 that play an essential role in autophagosome formation (Doelling et al., 2002; Slavikova et al., 2005; Suzuki et al., 2005; Fujioka et al., 2008; Geng and Klionsky, 2008; Phillips et al., 2008), and a phosphatidylinositol 3-kinase (PI3-kinase) complex (Liu et al., 2005). ATG8 is first cleaved by ATG4 protease to expose a C-terminal Gly residue then conjugated to phosphatidylethanolamine (PE) (Fujita et al., 2008) by ATG3 and ATG7 enzymes in a process similar to ubiquitination (Ichimura et al., 2000; Kirisako et al., 2000; Kim et al., 2001). ATG8-PE and ATG12-ATG5 conjugates (Mizushima et al., 1998) are required for initiation, expansion, and fusion of autophagosomal membrane with the vacuolar/lysosomal membrane (Hanada et al., 2007; Nakatogawa et al., 2007, 2009; Fujita et al., 2008; Geng and Klionsky, 2008; Xie et al., 2008). Additionally, other

enzymes are shared between autophagy pathways and other signaling cascades, such as the PI3-kinase domain-containing VPS34 (also termed ATG6 or Beclin 1 in mammals) which is located at the PAS and also involved in autophagosome formation (Duszenko et al., 2011). Downstream of *ATG* gene action is TOR (Target Of Rapamycin), which is a kinase that regulates the balance between anabolic and catabolic states of the cell (Blommaart et al., 1997; Dennis et al., 1999; Shigemitsu et al., 1999; Allison, 2000; Bonatti et al., 2000; Bursch, 2001; Mejillano et al., 2001; Duszenko et al., 2011). In yeast, mammals, and fruit flies, TOR has been identified as a negative regulator of autophagy (Díaz-Troya et al., 2008b) and more broadly regulates cellular processes, including growth and protein synthesis, in response to nutrient and growth factor availability (Blommaart et al., 1995; Noda and Ohsumi, 1998; Raught et al., 2001; Pattingre et al., 2008; Pérez-Pérez et al., 2010). TOR kinases are large proteins (270 kDa) that assemble into two different TOR-containing complexes of 1.5 to 2.0 MDa termed TOR complex 1 (TORC1) and TORC2 (Díaz-Troya et al., 2008a), each in control of a distinct signaling pathway (Duszenko et al., 2011). The TORC1 pathway mediates the rapamycin-sensitive signaling branch that perceives the nutritional status of the cell and controls processes such as ribosome biogenesis, transcription, translation, and induction of autophagy; while TORC2, on the other hand, is insensitive to rapamycin and controls actin cytoskeleton remodeling (Loewith et al., 2002; Jacinto et al., 2004; Sarbassov et al., 2004.; Díaz-Troya et al 2008a; Pérez-Pérez et al., 2010). In yeast, inhibition of TORC1 by rapamycin leads to autophagy activation through a mechanism that requires an ATG1/ATG13 complex which regulates autophagy downstream of TOR (Kamada et al., 2000; Bassham, 2009).

The unicellular photosynthetic soil algae *Chlamydomonas reinhardtii* synthesizes fatty acids principally for esterification into glycerol-based membrane lipids (Hu et al., 2008), but when deprived of nitrogen in liquid cultures, it slows down the cell proliferation and alters the lipid biosynthetic pathways towards the formation and accumulation of neutral lipids, mainly in the form of triacylglycerol (TAG) (Hu et al., 2008). This study reports the effects of autophagy-inducing (Rapamycin) and autophagy-inhibiting (3-Methyladenine) agents on the accumulation of TAGs in *Chlamydomonas reinhardtii* when subjected to nitrogen deprivation. Moreover, Pérez-Pérez et al. (2010) demonstrate that nutrient limitation triggers an autophagic response in *Chlamydomonas* cells that can be traced as an increase at the level of ATG8 protein, and that rapamycin treatment results in an induction of autophagy-like processes (Crespo et al., 2005; Díaz-Troya et al 2008b; Pérez-Pérez et al., 2010). ATG8 is a critical component of the autophagy pathway (Bassham, 2009). *Chlamydomonas ATG8* (*CrATG8*) is conserved and may be used as a specific autophagy marker (Pérez-Pérez et al., 2010).

Therefore, the main objective of this research is to determine if the TAG accumulation in nitrogen-deprived *Chlamydomonas* cells were due to an induction of autophagic pathways; we use rapamycin, an autophagy-inducer, and 3-methyladenine (3-MA), a powerful autophagy-inhibitor; followed by estimations of TAG contents.

Materials and Methods

Strains and culture conditions

Chlamydomonas reinhardtii strain CC-124 was used in all reported experiments. Cells were grown aseptically to the mid-logarithmic phase (~3 x 10^6 cells mL^{-1}) in tris-

acetate-phosphate (TAP) medium (Gorman and Levine, 1965) for cells grown mixotrophically or in high salt (HS) medium (Sueoka, 1960) for cells grown photoautotrophically in 500 mL Erlenmeyer flasks at 25 ˚C, under constant agitation (190 rpm), and continuous illumination with cool-white fluorescent bulbs at a fluence rate of 250 μmol m^{-2} s^{-1}. Culture growth was monitored using a spectrophotometer at 750 nm. Cells were harvested by centrifugation at 5000 rpm for 10 min, and used to inoculate culture tubes with fresh media with or without nitrogen at a starting density of 5 x 10^5 cells mL^{-1}; rapamycin was also added at different concentrations (0.5, 1, and 5 μM) to one set of culture tubes, and 3-MA (0.25, 0.5, and 1 mM) to another set of culture tubes. Cells were harvested by centrifugation and used for experiments described later.

Microscopy imaging of live cells and fluorescence intensity measurements

Equal cell number (~10^6) was harvested at different time points after incubation (0, 24, 48, 72, 144h) and stained with Nile Red (Sigma, St. Louis, Missouri, USA) at a final concentration of 0.5 μg mL^{-1} to visualize neutral lipids. Cell images were acquired with a fluorescent microscope (Olympus Fluoview 500) using 100X oil immersion lens. The peak of emission of Nile Red when associated with neutral lipids was close to 570 nm. The laser excitation line was at 443 nm and the emission signal was collected between 560 nm and 600 nm. The fluorescence intensity was also measured from cells incubated with 1 mM 3-MA and stained with Nile Red using Tecan Infinite® 200 series multimode microplate reader (Tecan Group Ltd., Switzerland); the excitation wavelength was at 530 nm and the emission signal was collected at 575 nm. Triolein was used as standard in samples quantifications.

Thin layer chromatography plate assay and gas chromatography analysis

Equal cell number (~1.5 x 10^7) was harvested after 6 days incubation in HS+N/-N media supplied with 1 mM 3-MA. For TAGs analysis, the total lipid extract was dried under nitrogen and resuspended in 100 μL of 6:1 (v/v) chloroform:methanol. This extract was applied to a silica 60 thin layer chromatography plate (Sigma-Aldrich) and neutral lipids were resolved using a solvent system of 70:30:1 (v/v/v) heptane:ethyl ether:acetic acid. The TAG band was identified by co-migration with a TAG standard extracted from soybean, stained with iodine vapors, in an adjacent lane. The TAG fraction was then recovered from the plate and lipids resuspended in 1.5 mL of 2.5% (v/v) sulfuric acid in methanol. Fatty acid methyl esters were prepared, extracted, and analyzed by gas chromatography (GC) using an Agilent 7890A gas chromatograph (Msanne et al., 2011). This yielded information on the TAG composition and the FA amounts. For GC analysis, triolein was used as an internal standard.

Results

Effects of rapamycin and 3-MA treatments on TAG accumulation

Rapamycin treatment (1μM) slows down the growth of *Chlamydomonas* cells, causes increased vacuolization, but does not induce neutral lipid accumulation when treated cells are grown for 6 days in nutrient replete (HS+N) and nitrogen-deprived (HS-N) media (Fig. 4.1A and 4.1B). Moreover, *Chlamydomonas* cells grown mixotrophically for 6 days in medium supplied with nitrogen (TAP+N), and inoculated with different rapamycin concentrations (0.5, 1, and 5 μM) show increased vacuolization but also no neutral lipid accumulation (Fig. 4.2).

On the other hand, treatment with different concentrations of 3-MA induces neutral lipid accumulation in *Chlamydomonas* cells grown in both nitrogen-deprived and nitrogen replete media (Fig. 4.3). Moreover, the fluorescence intensities measured using a Tecan microplate reader are significantly higher for cells incubated for 6 days in HS+N or HS-N and supplied with 1 mM 3-MA, indicating that cells treated with the autophagy-inhibitor accumulate higher amounts of neutral lipids (Fig. 4.4A). TAG contents, quantified using triolein as a standard, show an increase (in nanograms per 1000 cells) when measured in cells treated with 1 mM 3-MA (Fig. 4.4B).

Thin-layer chromatography and indirect quantification of TAG contents

Previous Nile Red staining experiments show TAG increase in *Chlamydomonas* cells after treatment with 3-MA. In the TLC plate assay, the separated lipid fractions are visualized by exposure of TLC plate to iodine vapors that forms a non-covalent brown complex with lipids. TLC plate assays show stronger bands with higher intensities when TAG band extracted from cells supplied with 1 mM 3-MA is compared to the band extracted from cells growing without 3-MA (Fig. 4.5).

Indirect quantification is the method of choice for the determination of TAG content in *Chlamydomonas* cells after incubation with 3-MA. Triacylglycerols are extracted from the adsorbent layer in the presence of an internal standard; distinct zones are carefully scrapped off, then transmethylated before analysis by gas chromatography of the methyl ester derivatives of the fatty acids. Data show an increase in triacylglycerol content in treated cells; TAG almost double in cells grown in HS-N and show a significant increase in cells grown in HS+N after treatment with 1 mM 3-MA (Fig. 4.6). The fatty acid composition of the purified TAG fraction is also determined (Table 4.1 and

Table 4.2). 3-MA treatment increases the individual fatty acids and this is reflected by the increase of the TAG content after treatment (Table 4.2).

Discussion

Autophagy is important for cell adaptation to changes in environmental conditions; it is a survival mechanism during nutrient deprivation, hypoxia, and high temperatures. Autophagy functions as a nutrient recycling pathway by removing damaged, redundant or unwanted cytoplasmic constituents including organelles; it also plays a major role in cellular remodeling. Autophagy pathways are studied in animals, plants, yeast, green algae, etc. and autophagy genes are conserved between species (Thompson and Vierstra, 2005; Bassham et al., 2006; Pérez-Pérez et al., 2010; Duszenko et al., 2011).

Previous studies indicate that depletion of various nutrients such as nitrogen, carbon, sulfur, or phosphorous triggers membrane reorganization, indicating that autophagy may be a physiological response to nutrient deprivation (Takeshige et al., 1992; Ohsumi, 2001). In this study, we analyze the effects of the autophagy-inducer rapamycin and the autophagy-inhibitor 3-MA on the accumulation of TAG in *Chlamydomonas reinhardtii* cells subjected to nitrogen deprivation. On the molecular level, rapamycin inhibits TOR signaling leading to autophagy induction. TOR kinase is a negative regulator of autophagy in *Chlamydomonas* and other eukaryotic cells; it is membrane-associated and has a role in autophagy in addition to growth regulation (Díaz-Troya et al., 2008a; Bassham, 2009; Pérez-Pérez et al., 2010). 3-MA, a powerful autophagy inhibitor, blocks autophagosome formation at a very early stage, and inhibits

the eventual cell death (Bursch, 2001; Bassham et al., 2009). At the molecular level, 3-MA inhibits PI3-kinase activity necessary for the nucleation of preautophagosomal structures (Petiot et al., 2000; Bursch, 2001; Thompson and Vierstra, 2005; Poustka et al., 2007). We conclude that the increase in neutral lipids in cells subjected to nitrogen deprivation may not be an autophagic response because the use of 3-MA still leads to a significant increase in TAG contents.

Non-aqueous thin-layer chromatography (TLC) assay allows direct visualization of TAG bands. TLC assays provide conclusive results regarding the size and intensity of the TAG bands and the amount that increases significantly after inoculation with 3-MA (Fig. 4.5). *Chlamydomonas* cells growing on media containing nitrogen and supplied with 3-MA also show slight TAG induction ccompared to control cells (Fig. 4.5). The retention of the *Chlamydomonas* lipids on the TLC plate when the solvent system of 70:30:1 (v/v/v) heptane:ethyl ether:acetic acid is used increases in the following order: chlorophyll A and B (Bottom), monoacylglycerols, diacylglycerols, free fatty acids, triacylglycerols, carotenoids (Top) (Fig. 4.5).

Indirect quantification of the fatty acid methyl esters extracted from the TLC plate indicates that the TAG content almost doubles in cell incubated for 6 days in HS-N medium supplied with 3-MA (Fig. 4.6 and Table 4.2). Moreover, 3-MA induces TAG accumulation in cells grown in HS+N medium supplied with 3-MA (Fig. 4.6 and Table 4.2).

Rapamycin-mediated inhibition of TORC1 pathway results in increased CrATG8 modification and protein relocalization in *Chlamydomonas* cells; moreover, a significant increase in CrATG8 protein abundance is detected in cells growing under nutrient-

deprived conditions (Pérez-Pérez et al., 2010). Therefore, *CrATG8* has a prominent role in the control of autophagy in *Chlamydomonas reinhardtii*. Moreover, *Chlamydomonas* genome has a single *ATG8* gene that spans about 2 kb of genomic DNA and consists of five exons and four introns; CrATG8 protein has 134 amino acids and a molecular mass of 15 kD (Díaz-Troya et al., 2008b).

We have examined whether a tandem inverted repeat construct can trigger the downregulation of *CrATG8* gene which is conserved and used as an autophagy marker. Downregulation of *CrATG8* may lead to autophagy suppression. We expect the RNAi *Chlamydomonas* mutants that are defective in *CrATG8* expression to behave as if the cells were treated with the autophagy-inhibitor 3-MA; these cells may accumulate higher contents of neutral lipids when subjected to nitrogen deprivation. However, results of these experiments are not yet available.

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Figures

Figure 4.1 Rapamycin treatment of *Chlamydomonas reinhardtii* CC-124 subject to nitrogen deprivation. Cells were cultured for 6 days in High Salt medium (HS+N) or in the same medium lacking nitrogen (HS-N). (A) Fluorescence microscopy detection of neutral lipid accumulation by Nile Red staining. (B) Fluorescence microscopy detection of cells treated with 1 μM rapamycin. The upper panels correspond to merged transmitted light and fluorescent images whereas the lower panels correspond to fluorescent images. Scale bar equals 10 μm.

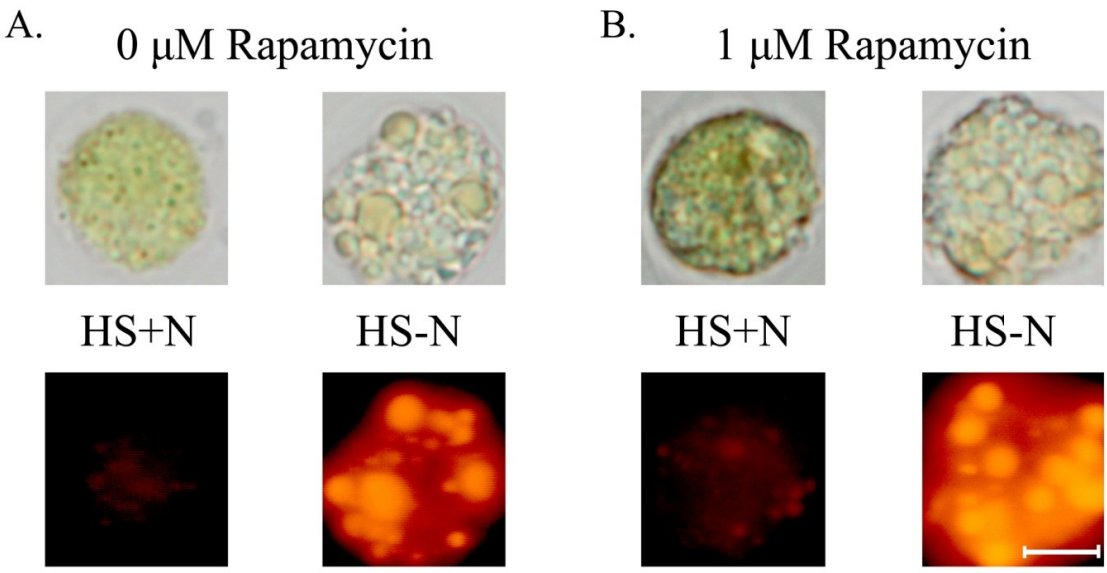


Figure 4.2 Fluorescence microscopy detection of *Chlamydomonas reinhardtii* CC-124 cells cultured for 6 days in Tris-Acetate-Phosphate medium (TAP+N) and treated with different rapamycin concentrations. (A) CC-124 control cells. (B) CC-124 cells plus 0.5 μM rapamycin. (C) CC-124 cells plus 1 μM rapamycin. (D) CC-124 cells plus 5 μM rapamycin. The upper panels correspond to merged transmitted light and fluorescent images whereas the lower panels correspond to fluorescent images. Scale bar equals 10 μm.

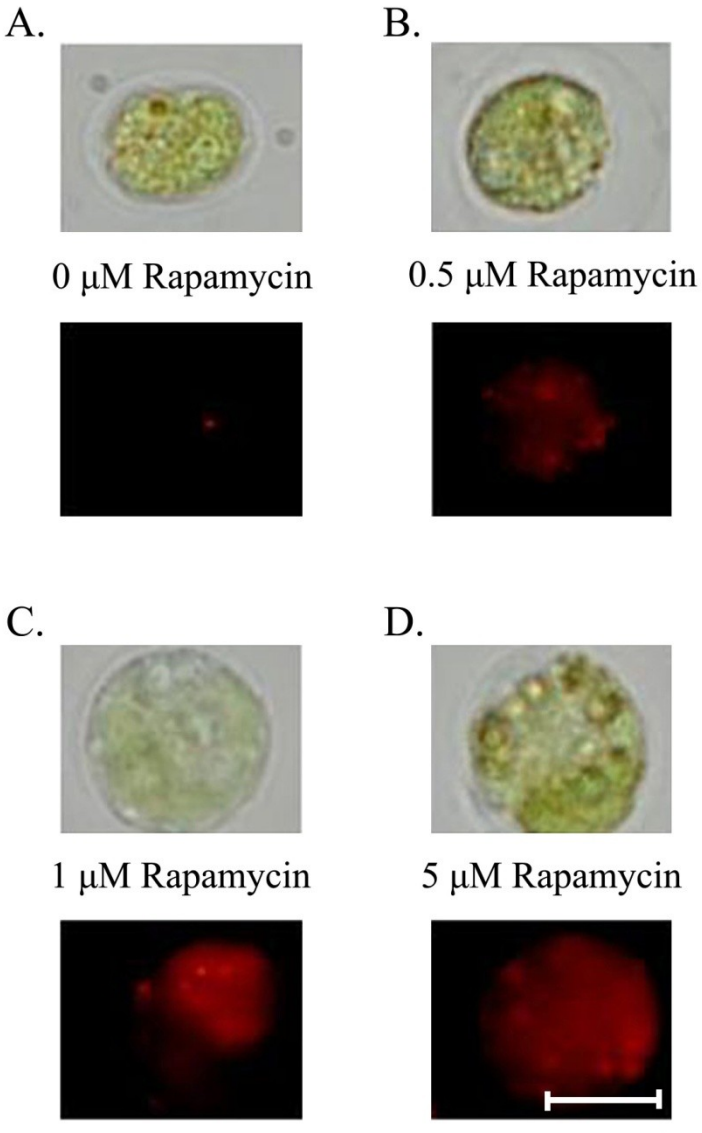


Figure 4.3 3-Methyladenine (3-MA) treatment of *Chlamydomonas reinhardtii* CC-124 subject to nitrogen deprivation. Cells were cultured for 6 days in HS+N or HS-N. (A) Fluorescence microscopy detection of neutral lipid accumulation in control CC-124 cells. (B) Fluorescence microscopy detection of cells treated with 0.25 mM 3-MA. (C) Fluorescence microscopy detection of cells treated with 0.5 mM 3-MA. (D) Fluorescence microscopy detection of cells treated with 1 mM 3-MA. The upper panels correspond to merged transmitted light and fluorescent images whereas the lower panels correspond to fluorescent images. Scale bar equals 10 µm.

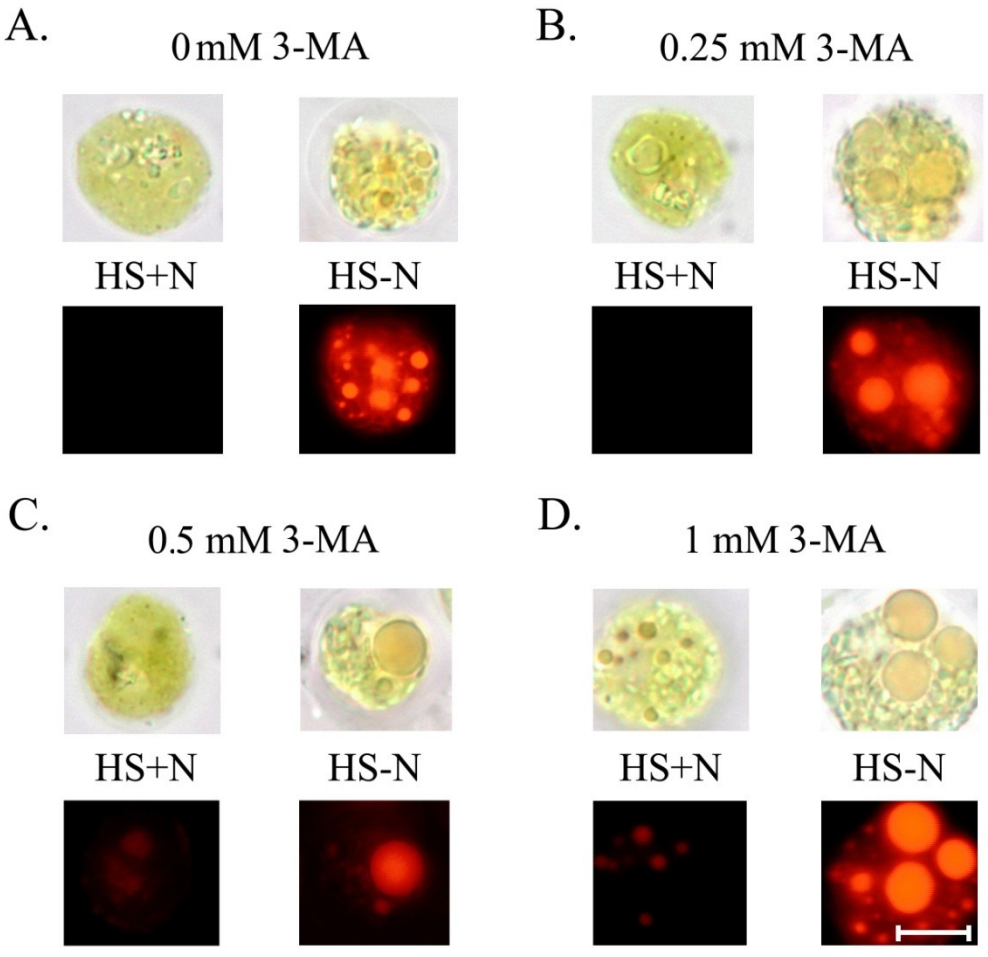


Figure 4.4 Fluorescence intensity measured from *Chlamydomonas reinhardtii* CC-124 incubated with 1 mM 3-MA and subject to nitrogen deprivation. Cells were stained with Nile Red. (A) Fluorescence intensities measured from cells subjected to various treatments at the indicated times. (B) Amounts of neutral lipids (in nanograms per 1000 cells) calculated from cells subjected to various treatments at the indicated times. Triolein was used as a standard in sample quantifications. Each data point represents the average of three independent experiments ± s.e.

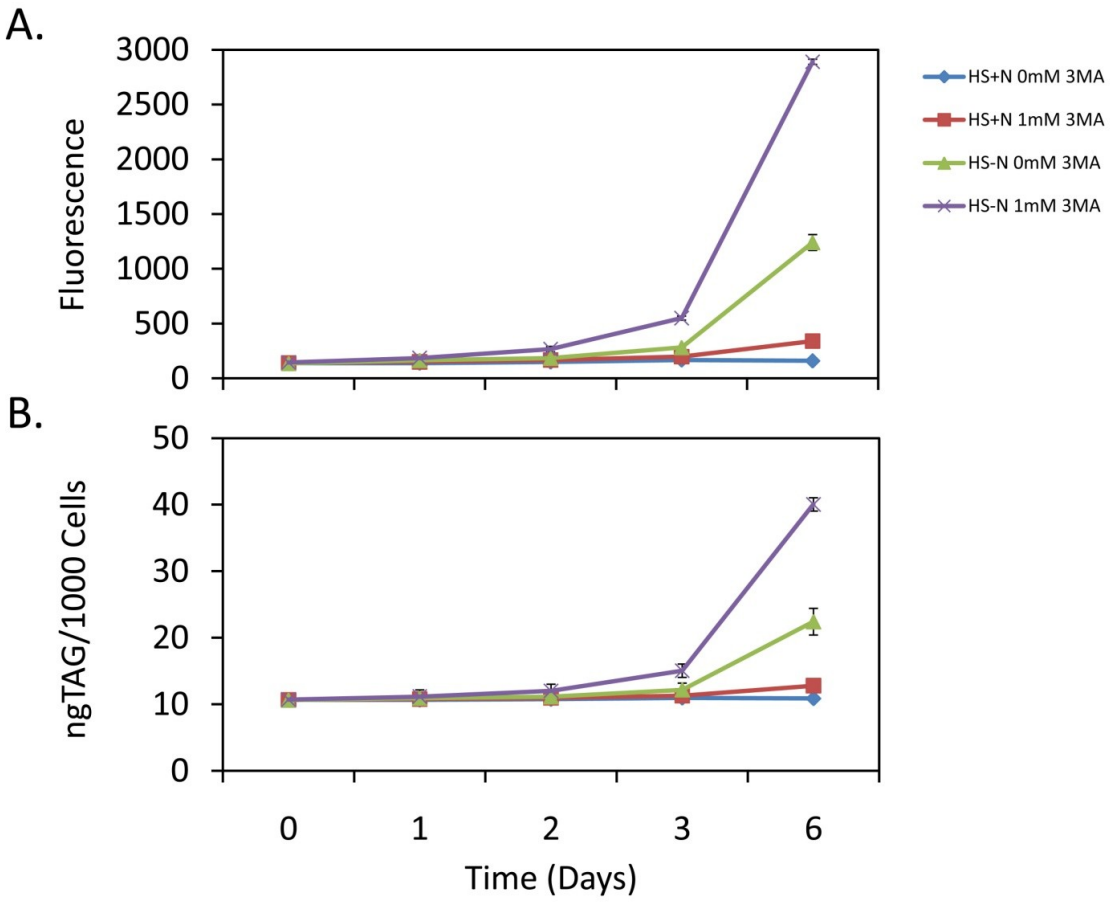


Figure 4.5 Thin layer chromatography (TLC) assay used to separate different lipid fractions to visualize intensities of the TAG bands extracted from *Chlamydomonas reinhardtii* cultured for 6 days in HS+N or HS-N and subjected to treatments with 1mM 3-MA. Soybean TAG is used as control.

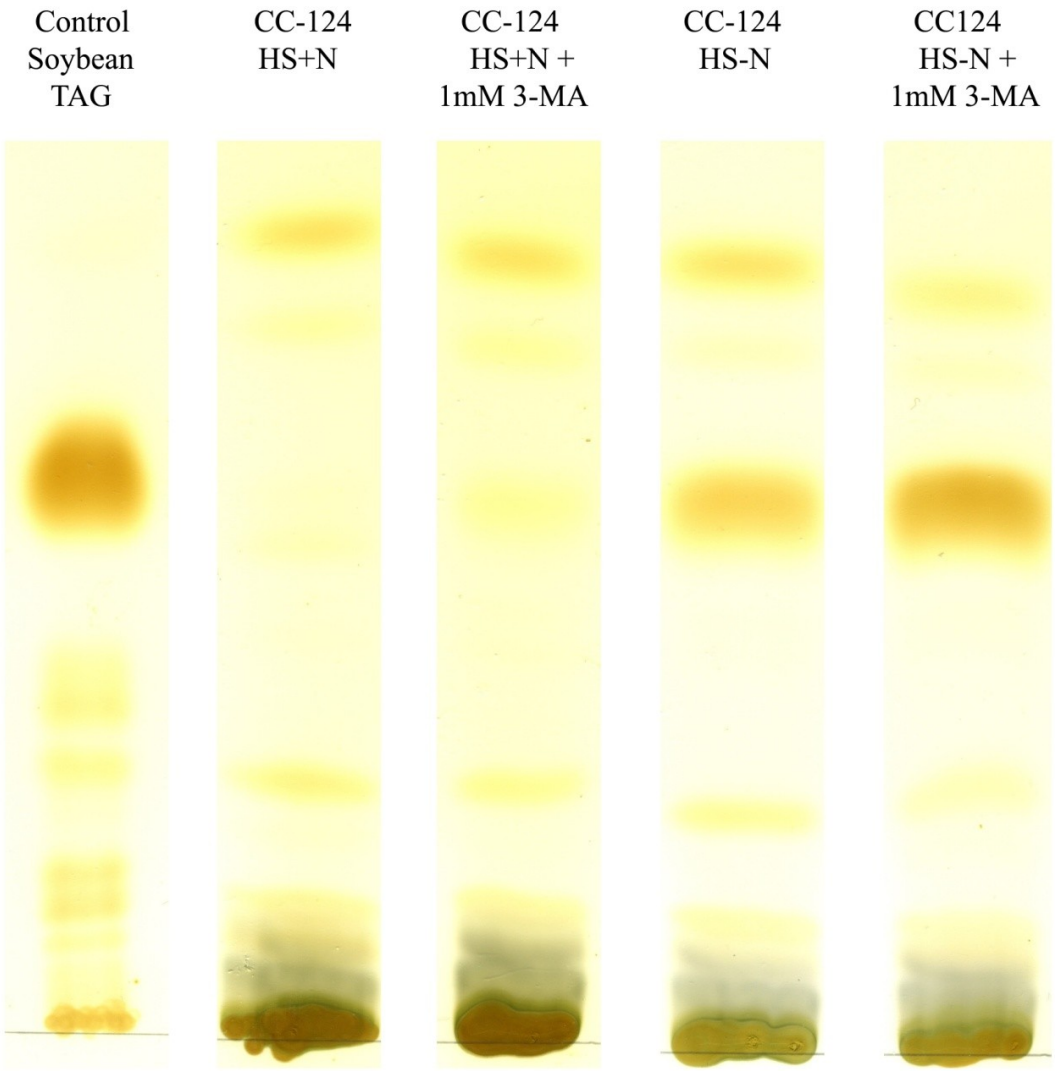
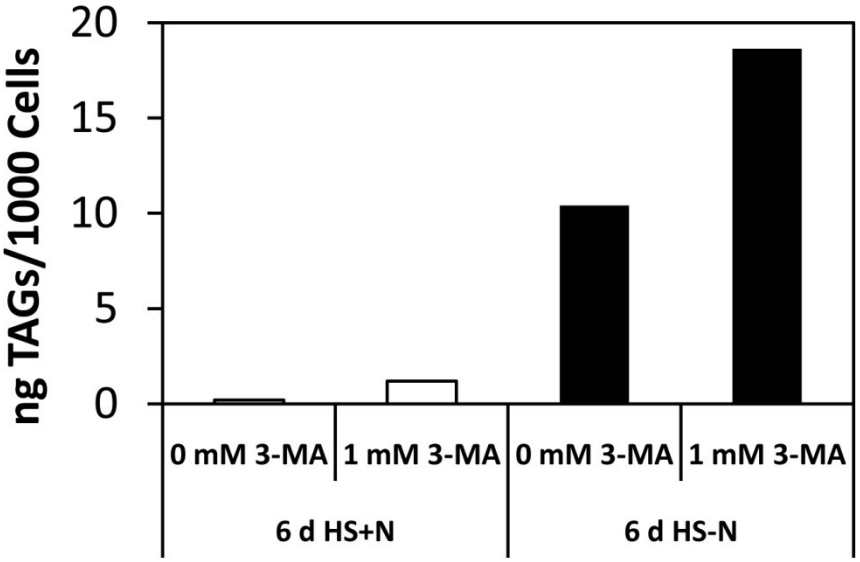


Figure 4.6 Triacylglycerol contents (nanograms per 1000 cells) in *Chlamydomonas reinhardtii* CC-124 incubated with 1 mM 3-MA and subject to nitrogen deprivation. Cells were grown for 6 days in HS medium either nutrient replete (+N) or nitrogen depleted (-N). Values correspond to the average of two independent experiments.



Tables

Table 4.1 Fatty acid composition (wt%) of the purified triacylglycerols (TAGs) from *C. reinhardtii* cells cultured in HS-N (± 1mM 3-MA) media for 6 days.

Sample Name	16:0	16:1	16:3	16:4	18:0	18:1 Δ9	18:1 Δ11	18:2	18:3 Δ5,9,12	18:3	18:4 Δ5,9,12, 15	Other
6 d HS-N	29.5	4.1	2.4	4.9	2.4	18.9	5.4	13.8	4.9	10.1	1.7	1.9
6 d HS-N 3-MA	27.3	6.7	3.0	4.5	2.6	18.0	7.4	14.0	3.0	9.9	1.2	2.5

Table 4.2 Content of fatty acids (FAs) in purified TAGs from *C. reinhardtii* cells maintained in HS+N or HS-N (± 1mM 3-MA) media for 6 days. Values shown are expressed as ng FA/1000 cells.

Sample Name	ng TAG/1000 cells	16:0	16:1	16:3	16:4	18:0	18:1 Δ9	18:1 Δ11	18:2	18:3 Δ5,9,12	18:3	18:4 Δ5,9,12, 15	Other
6d HS+N	0.19	0.12	0.02	0	0	0	0.06	0	0	0	0	0	0
6d HS+N 3-MA	1.19	0.56	0.04	0	0	0.04	0.27	0.04	0.08	0.02	0.04	0.06	0.03
6d HS-N	10.41	3.07	0.42	0.25	0.51	0.25	1.97	0.56	1.44	0.51	1.05	0.17	0.20
6d HS-N 3-MA	18.63	5.09	1.25	0.55	0.84	0.49	3.36	1.38	2.60	0.56	1.84	0.22	0.46

CHAPTER 5 – CONCLUSIONS

There are extensive connections among cold, drought, and salinity signal transduction pathways (Ishitani et al., 1997; Xiong et al., 2002). *RD29A* and *RD29B* genes are stress-inducible and not expressed under normal conditions; the proteins encoded by these two genes remain uncharacterized, they have resemblance to late embryogenesis abundant (LEA) proteins and hence are called LEA-like proteins. Possible utility of *RD29A* and *RD29B* genes for conferring abiotic stress resistance in plants is partly examined in this research. Transformation of *rd29a* and *rd29b* knockout mutant lines using chimeric gene constructs with the native promoters swapped show that the promoter of *RD29A* is responsive to cold, drought, and salt stresses, whereas the promoter of *RD29B* is highly responsive to salt stress. In dry regions, overcoming salt stress is a main issue to ensure agricultural sustainability and crop production (Meloni et al., 2004). Salinity causes a significant ($p < 0.05$) reduction in root and shoot dry biomass (Meloni et al., 2004). Previous studies carried out with cotton (Meloni et al., 2001), as well as with soybean and alfalfa (Berstein and Ogata, 1966; Kant et al., 1994) also show that shoot growth is more inhibited by NaCl than root growth (Meloni et al., 2004). Upstream promoter sequences of *RD29A* and *RD29B* might be useful to confer distinct abiotic stress resistance in some plants by driving expression of transcription factors that control stress ‘‘regulons’’. Kasuga et al. (1999) report that overexpression of the transcription factor *DREB1A* driven by the stress-inducible *RD29A* promoter is able to significantly improve plant tolerance to cold, drought, and high salinity with plant growth and seed production similar to wild-type plants under normal growing conditions. *RD29A* promoter is able to minimize the negative effects on plant growth experienced with use of

the *35S CaMV* promoter. Despite the ability of the promoters to confer inducible upregulation of stress responses pathways and thus protection against dehydration, *rd29a* and *rd29b* knockout mutant lines maintain greater root growth, photosynthesis (A), and water use efficiency (WUE) under salt stress relative to control. Therefore, RD29A and RD29B proteins are unlikely to serve directly as protective molecules but may function as warning signals for abiotic stress responses. Further studies are needed to better understand protein roles and different stress batteries (i.e. UV light, ROS, etc.) may give a better idea regarding the RD29 protein functions. Moreover, for different experiments performed in this analysis, cold, drought, and salt are applied for a short time; it will be interesting to study the roles of RD29A and RD29B proteins under prolonged stress and analyze the phenotypes of T-DNA knockout mutant plants.

Whether conditions are favorable or adverse, the intricate metabolic pathways of a cell are heavily influenced by its environment (Rosenberg et al., 2008). In the case of microalgae, nitrogen deprivation stimulates dramatic changes in metabolism. In *Chlamydomonas reinhardtii*, cells growing photoautotrophically and subjected to N deprivation initially accumulate starch, which increases its content ~14-fold during the first two days of stress. *Chlamydomonas* cells also show a significant increase in triacylglycerol (TAG) content, representing ~70% of the total FAs, after 6 days of N deprivation. These observations suggest considerable *de novo* fatty acid synthesis in cells depleted of nitrogen for several days, but the synthesized lipids are unlikely to derive from newly assimilated carbon, since chlorophyll and total protein contents per cell are greatly diminished at these time points. Nitrogen deprivation triggers similar changes in starch, TAG, and chlorophyll content in *Coccomyxa* sp. C-169, suggesting that these

metabolic patterns may be shared by other green microalgae. Genetic manipulation can enhance TAG accumulation in microalgae; Wang et al. (2009) report a 15-fold increase in TAG content when starch biosynthesis is blocked in the *Chlamydomonas reinhardtii sta6* mutant defective in ADP-Glucose pyrophosphorylase that catalyzes the rate-limiting step in starch formation by converting Glucose-1-phosphate to ADP-Glucose. Semiquantitative RT-PCR analyses support an early recycling of FAs into TAGs since at least two genes encoding DGATs, catalyzing the terminal acylation of diacylglycerol into TAG, are rapidly upregulated. *KASI* and *KAR* are found to decrease in expression under N deprivation. Overexpression of *KASI* which is involved in fatty acid elongation may also lead to an increase in TAG accumulation since the FA biosynthesis pathway is douwnregulated by nitrogen deprivation.

The gene regulatory systems of microalgae, determining these changes, remain unexplored. Several microRNAs and transcription factors increase in abundance under nitrogen starvation in *C. reinhardtii*. A greater understanding of these regulatory systems may provide targets for the metabolic engineering of algal strains with enhanced accumulation of biofuel precursors. Sequencing of total RNA extracted from *C. reinhardtii* cells growing under nitrogen-deprived conditions and transcriptome analysis may provide information on differential gene expression.

TAG accumulation and induction of autophagic pathways in *Chlamydomonas reinhardtii* cells, growing in nitrogen-deprived media, is also investigated. We use rapamycin, which induces autophagy by inhibition of TORC1 pathway, a negative regulator of autophagy, and 3-methyladenine (3-MA), a powerful autophagy-inhibitor that blocks autophagosome formation. 3-MA inhibits PI3-kinase activity. Rapamycin

treatment has no effect on TAG accumulation when *Chlamydomonas* cells are grown in high salt (HS) media lacking nitrogen. On the other hand, treatment with different concentrations of 3-MA significantly increases TAG accumulation in cells grown in both nitrogen-deprived and control media. Because 3-MA inhibits autophagy, the increase in TAG accumulation in cells grown in media lacking nitrogen may not be a direct response to autophagy induction by nutrient depletion. Nitrogen deprivation and rapamycin treatment lead to CrATG8 modification and an increase in the protein level. *CrATG8* is under the control of the rapamycin-sensitive TORC1 signaling pathway and is a specific autophagy marker induced early in the autophagy signaling pathway; therefore, downregulating the expression of the *CrATG8* gene by RNA interference may theoretically block autophagy induction. We expect RNAi mutants showing effective *CrATG8* silencing to behave in a similar way to cells treated with 1 mM 3-MA when growing under nitrogen-limiting conditions.

Water is becoming an increasingly precious commodity and population growth is also contributing to the need to use marginal lands for agricultural production, increasing the exposure of crop plants to high salinity. Therefore, it is imperative to find new approaches to develop stress-resistant crops to maximize yields. The cold, drought, and salt-stress responsive *Arabidopsis thaliana RD29A* and *RD29B* genes (and their upstream promoter sequences) can be exploited for conferring abiotic stress resistance in some crop plants. In this research, we generate fundamental knowledge about these gene sequences, and determine their possible utility in genetic engineering to improve crop performance. On the other hand, renewable “green” biofuels are needed to displace petroleum-derived fuels, which contribute to global warming and are of limited availability. The use of

microalgae can be a suitable alternative feedstock for next generation biofuels; they have fast growth rate, permit the use of non-arable land and non-potable water, and do not affect the supply of food and other crop products. The interest in microalgae for oil production is due to the high lipid content, which is of great importance for the food and energy industries. In this study, we also generate fundamental information about the genetic and biochemical mechanisms associated with the induction of oil synthesis in *Chlamydomonas reinhardtii* and *Coccomyxa* sp. C-169, under nitrogen deprivation conditions, when grown in liquid media.

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